UNIVERSITY
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THE APPLICATION OF NOVEL EXTRACTION AND ANALYTICAL TECHNIQUES IN FORENSIC TOXICOLOGY

Thesis submitted in Accordance with the Requirements of the University of Glasgow for the Degree of Doctor of Philosophy

By

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Joseph Black Building
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To

Pa and Ma

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AIBN	2,2'-Azobisisobutyronitrile
APCI	Atmospheric Pressure Chemical Ionisation
API	Atmospheric Pressure Ionisation
EGDMA	Ethylene glycol dimethacrylate
ELISA	Enzyme-linked Immunosorbent Assay
ESI	Electrospray Ionisation
FAB	Fast Atom Bombardment
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GLC	Gas Liquid Chromatography
HFBA	Heptafluorobutyric Acid
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
LC-ESI-MS	Liquid Chromatography-Electrospray Ionisation-Mass Spectrometry
LC-MS	Liquid Chromatography-Mass Spectrometry
LC-MS-MS	Liquid Chromatography With Tandem Mass Spectrometry
LLE	Liquid-Liquid Extraction
LLOQ	Lower Limit Of Quantification
LOD	Limit Of Detection
LOQ	Limit Of Quantification
MAA	Methacrylic Acid
MIP	Molecularly Imprinted Polymer
MISPE	Molecularly Imprinted Solid-Phase Extraction
MS	Mass Spectrometry
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NIP	Non-imprinted Polymer
PFPA	Pentafluoropropionic Acid
QA	Quaternary Ammonium
RF	Radio Frequency

RIA	Radioimmunoassay
RPLC	Reverse-Phase Liquid Chromatography
RSD	Relative Standard Deviation
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscope
SPE	Solid-Phase Extraction
TFA	Trifluoroacetic Acid
TFMAA	Trifluoromethacrylic Acid
UV	Ultraviolet

SUMMARY

The aim of this study was to investigate new methods of analysis which might be applied to forensic toxicology problems including those resulting from pesticides, particularly the quaternary ammonium herbicide group, and from drugs, particularly the benzodiazepine group.

In the first part of this study, an efficient method for the determination of quaternary ammonium (QA) compounds (pesticides and drugs) in human whole blood was developed. Atracurium, bretylium, edrophonium, ipratropium, mivacurium, neostigmine, pancuronium, and rocuronium (QA drugs) and difenzoquat, diquat and paraquat (QA pesticides) were analyzed by LC-MS-MS. The target analytes were extracted with Bond Elut® LRC-CBA cartridges from whole blood samples containing internal standard. Internal standards for drug and herbicide analysis were benzyldimethylphenylammonium chloride monohydrate and ethyl viologen, respectively. The liquid chromatographic separation of the QA compounds was carried out on a Atlantis® dC18 column (100 mm x 2.1 mm ID, 5 μm) and guard column (2.0 mm x 2.1 mm, 5 μm) using ion-pair chromatography with heptafluorobutyric acid (15 mM)/ammonium formate (20 mM) buffer adjusted to pH 3.30 with formic acid and methanol to establish a gradient elution. The elution program consisted of a linear gradient from 5-90% of methanol within 18 minutes. Mass spectrometry was conducted in the positive ion electrospray ionization (ESI) mode. An ion transfer capillary temperature of 275°C was employed. The nitrogen sheath and auxiliary gas flow rates were set at 15 and 5 arbitrary LCQ units respectively.

Method validation found that the recoveries of QA compounds ranged from 79.7-105.1%. The limits of detection (LODs) were between 3.6-20.4 ng/mL and lower limits of quantification (LLOQs) were in the range between 12.1-68 ng/mL. The intra- and inter-day precisions were equal to or less than 18.6% at a concentration of 10 ng/mL. The method was applied to three cases of paraquat poisoning, in which the concentrations of paraquat in blood (two cases) and plasma (one case) were within the range associated with fatal paraquat poisoning.

The second part of this study concerned the development of a novel sorbents for solid phase extraction using molecularly imprinted polymers (MIPs). The synthesis of an MIP involves the addition of the target analyte as a template molecule to a mixture of functionalized monomers and cross-linkers which then orientate round the template with

selective coordination. After polymerization, the template is extracted with solvent and what remains is a recognition site which is complementary in shape and functionality to the template structure. This recognition site is able to rebind with the template or an analogue molecule. In this study, the approach adopted was initially to synthesise a known MIP using diazepam as template then to prepare novel MIPs using other benzodiazepines and analogues of QA compounds as templates.

In the first of these stages, an anti-diazepam MIP was synthesized using methacrylate acid (MAA) as the monomer and ethylene glycol dimethacrylate (EGDMA) as the cross-linker and was then ground and prepared for use as an SPE sorbent by packing it into SPE cartridges. These cartridges were used to clean up extracts of diazepam and other benzodiazepine drugs made from hair samples via a molecularly imprinted solid-phase extraction (MISPE) protocol. Optimum retention of diazepam on the MIP columns was achieved using an apolar solvent, toluene, to load the extracts on the cartridges. The binding capacity of the polymer towards diazepam was 110 ng of diazepam/ mg of polymer. Analytes were eluted using 15% (v/v) acetic acid in acetonitrile. Chromatographic separation of the extracts was performed using Gemini C18 column (150 mm x 2.0 mm ID, 5 μm) and guard column (4.0 mm x 2.0 mm ID, 5 μm). HPLC was carried out using 3mM ammonium formate and acetonitrile. The elution program consisted of a linear gradient from 65-20% of 3 mM ammonium formate for 13 minutes. From 13.5 to 16.5 minutes, the percentage of 3 mM ammonium formate was 10% before increased back to initial conditions until 3.5 minutes. 20 µl of samples were injected into Gemini C18 column at a flow rate of 0.3 ml min^{-1}. Ionisation of analytes was performed using electrospray ionisation (ESI) on the positive mode.

The recovery of diazepam in a blank hair spiked at a concentration of 50 ng/mg using 30 mg hair was 93% with good precision (RSD = 1.5%). LOD and LLOQ of diazepam in spiked hair samples were 0.09 ng/mg and 0.14 ng/ mg using 30 mg hair, respectively. The MISPE method was also found to be applicable to the analysis of diazepam metabolites and other benzodiazepine drugs in addition to diazepam itself. The application of the extraction method to post-mortem hair samples yielded results that were in good agreement with ELISA data (from blood samples) and data arising from the analysis of the same blood samples using a validated in-house SPE-LC-MS-MS method.

The MISPE procedure was also compared with a conventional SPE method for analysis of benzodiazepines in hair samples. The results from MISPE protocol showed better

selectivity, specificity and accuracy toward diazepam (template molecule) and other benzodiazepines that display a similar resemblance to diazepam in terms of molecular structure. The MISPE procedure was found to be simpler and to offer cleaner extracts compared to a conventional SPE method.

An anti-flunitrazepam MIP was also synthesized and applied to analysis of hair samples. Unlike diazepam MIP, it was not possible to remove completely the template from flunitrazepam MIP. To avoid the problem which arises due to the bleeding of the template from the MIP during the extraction, which may lead to a false positive result, the MISPE procedure was applied to other benzodiazepines apart from flunitrazepam and 7-aminoflunitrazepam. The results were similar for most of the benzodiazepines when compared to the diazepam MISPE. However, the affinity of the diazepam MIP for benzodiazepines was found to be better than the flunitrazepam MIP.

In general, both diazepam and flunitrazepam MISPE methods displayed good sensitivity and selectivity, indicating that this method is sufficiently accurate, precise and reproducible to be used for trace benzodiazepine analysis in hair samples.

Finally, two anti-paraquat MIPs were synthesized using different analogue molecules (isomers of dipyridine) as templates to avoid template bleeding issues and also to have templates which would dissolve in the polymerization solvent (chloroform). However, the selectivity of both MIPs toward paraquat was very low due to poor binding efficiency of the imprinted polymers, therefore no further experimental work was carried out on these. The reason for this finding remains uncertain, but it is most probably due to the reaction of the mixtures used in the synthesis of the polymers. In order to obtain a good imprinted polymer, many factors need to be considered including the selection and composition of the monomer and cross-linker and the polymerization conditions.

1 GENERAL INTRODUCTION

1.1 Forensic Toxicology

'All substances are poisons; there is none that is not a poison. The right dose differentiates a poison from a remedy'

Paracelcus (1493-1541)

Poisons, as described in this famous quotation by Paracelcus, are substances that can be lethal when introduced into or absorbed by living organism in sufficient quantity [1]. Even harmless pure water can cause incapacitating electrolyte imbalance or even death if ingested in sufficient quantity [2]. Toxicology, or the study of poisons, had begun by 1500 BC [2, 3]. It is concerned with the chemical and physical properties of poisons, their symptoms and treatments and the identification of poisons in biological and non-biological materials.

Forensic toxicology is the branch of toxicology that is concerned with medico-legal aspects of the harmful effects of chemicals or poisons. It is the study of chemical composition, preparation and identification of poisons, commonly alcohol, drugs and chemicals. It is also includes knowledge about the absorption, distribution and elimination characteristics of such substances in the body [2].

1.2 Geographic Variations in Poisoning

The types of poisons routinely encountered by toxicologists may differ from one region of the world to another.

In the Western region, problems with alcohol abuse have been encountered for many years and alcohol has long been recognised as a major contributor to suicides. From the data reviewed by Room *et al.*, the alcohol-related burden of disease in the developed countries is 12.1% compared to 1.3% in the developing countries [4]. However, as part of the contemporary dynamic of globalisation, an increasing number of alcohol-related problems have recently been observed in developing countries [4].

In the developed world, the non-drug poisons which are routinely detected include solvents, which are a significant cause of death in young people. However, in the developing countries, it is pesticides which have generated large-scale fatal poisoning cases. It was reported that pesticide poisoning is the commonest form of fatal self-harm in rural areas in the developing countries [5]. The number of occupational and accidental pesticide poisoning cases is also high in the developing countries due to several factors such as the impracticality of safety equipment in humid countries, illiteracy among farmers who are unable to understand the instructions on containers, and lack of facilities for storage and disposal. The herbicide paraquat is one of the most common causes of substance-related death in South East Asia, including Malaysia. A similar situation existed in the United Kingdom in the 20th century until the agricultural formulation, Gramoxone®, was taken off the market.

It has also been reported that accidental poisoning in children in industrialised countries is due to ingestion of common, and relatively safe, medications whereas a far higher accidental death rate occurs amongst children in developing countries due to toxic pesticide poisoning [5].

Interestingly, both regions are dealing with the same weight of drug problems. The only differences are in the types of drugs of abuse. The global trends in illicit drug use and trafficking are well summarised by Cone *et al.* [6]. In Asia, cannabis and opium are popular and largely cultivated in the region. Recently, increases in the abuse of pharmaceutical preparations such as buprenorphine and diazepam have been reported. It is expected that drug abuse will be one of the leading causes of sickness in Asia by 2020 [7]. For comparison, in the United States alone, 30,000 premature deaths per year have been estimated to be due to the abuse of illegal drugs [6].

Currently, the development of scientific techniques for the analysis of drug and non-drug substances is important and advancing rapidly.

1.3 Recent Trends in Forensic Toxicology Analysis

Analytical techniques for the analysis of substances have been developed for various types of biological matrices. Recently the use of solid phase extraction (SPE) methods for isolation of analytes from biological matrices has been routinely applied. This extraction procedure is simple and reproducible and has reduced the volume of organic solvents used in the liquid-liquid extraction (LLE) process. Solid phase extraction in itself began in the mid 20th century using adsorbents such as Amberlyte XAD-2 resin, a polystyrene material, but its present format based on substituted silica materials (Figure 1-1) began in the 1980's, originally through the work of Professor Michael Burke of Arizona University and then through the first commercial supplier, Analytichem [8].

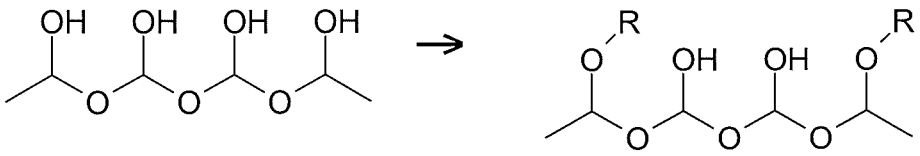


Figure 1-1 Preparation of substituted silicas as SPE sorbents

Apart from proliferation of companies manufacturing look-alike products, little changed for more than 10 years in SPE since the initial product range was introduced, apart from introduction of mixed-mode sorbents such as Certify™. In the last few years, polymeric sorbents have been introduced such as Strata™ X which has the structure shown in Figure 1-2, which have the advantage of having no residual surface silanol groups, unlike the substituted silica sorbents. Other relatively recent developments have included solid phase micro extraction (SPME), which uses a coated silica needle for extraction.

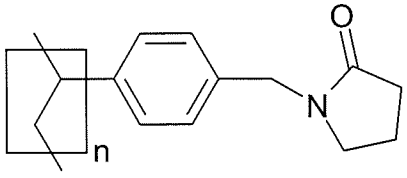


Figure 1-2 Structure of Strata™ X

SPE sorbents have also shown many advantages over LLE in terms of selectivity, for example, extraction of basic drugs using a mixed-mode sorbent gives extracts with few interferences. However, alternative approaches have been developed to improve selectivity, including the synthesis of sorbents loaded with antibodies (antibody mediated extraction) and molecularly imprinted polymers, which are essentially synthetic molecules with antibody-like specificity. The latter were investigated in the work for this thesis.

During the past century, chromatography has been the most notable analytical technique applied to the analysis of forensic biological samples. Its various formats have included paper chromatography, thin layer chromatography, gas chromatography and liquid chromatography. Combined techniques which dynamically linked gas chromatography to spectrometric methods including mass spectrometry and infrared spectrometry appeared from the 1960's onwards. High performance liquid chromatography began about the same time but its development was delayed due to technical difficulties, which were largely solved by the 1980's. The need for a similar practical interface between HPLC and mass spectrometry was recognised early on but many technical problems existed in the interface [9, 10]. The development of a usable LC-MS technique generated great interest among forensic toxicologists. The number of publications of LC-MS methods increased exponentially, starting from 1990, approaching the number of GC-MS publications per year in the mid-1990s and almost doubling it from 2003 on [11]. This method has been a valuable tool for routine analysis of forensic samples. The theory of SPE and LC-MS will be further discussed in the following chapters.

1.4 Aims of the Project

The work described in this thesis was designed with a specific purpose in mind, that is, to investigate modern methods of analysis and how these might be applied to the forensic toxicology problems of Malaysia. The initial aim was to target quaternary ammonium herbicides (including paraquat) but this was extended to include other quaternary compounds which would be co-extracted and co-eluted by the method. Subsequently, the recently-developed molecularly-imprinted polymer technique was investigated as a means of obtaining selective extraction of these substances from biological samples.

2 QUATERNARY AMMONIUM COMPOUNDS

2.1 Introduction

Quaternary ammonium (QA) compounds contain a functional group that consists of an ammonium group in which the four hydrogens are substituted by organic radicals (Figure 2-1). As a result, the nitrogen atom carries a permanent positive charge which cannot be neutralised by adjusting the pH in solution and which is associated with a negative counter-ion. This functional property tends to dominate the chemistry of quaternary ammonium compounds and also their methods of analysis.

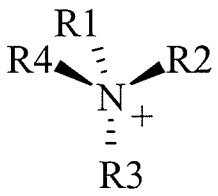


Figure 2-1 General molecular structure of QA

QA compounds have been used for over 50 years in the agricultural sector as herbicides [12] and in the medical area as neuromuscular blockers [13]. QA compounds have also been used extensively throughout the healthcare, water treatment and food processing industries as microbiocides and surfactants due to their non-corrosive nature and cleaning ability. Their salts also provide exchange sites on certain anion exchange resins.

This study focuses on QA compounds that have been used as herbicides and drugs which are also found to be abused and encountered in poisoning cases, due to their high toxicity level.

2.1.1 Quaternary Ammonium Herbicides

QA herbicides have been used to fight weeds that may interfere with land management systems. Weeds can also serve as alternative host for many insects and plant pathogens which subsequently lead to loss of yield. QA herbicides have been widely used in

developing countries for many years. In Malaysia, an average of 200 positive paraquat poisoning cases was reported every year in the 1990's [14]. Although it has been banned in Malaysia since 2003, the numbers of poisoning cases have not significantly decreased.

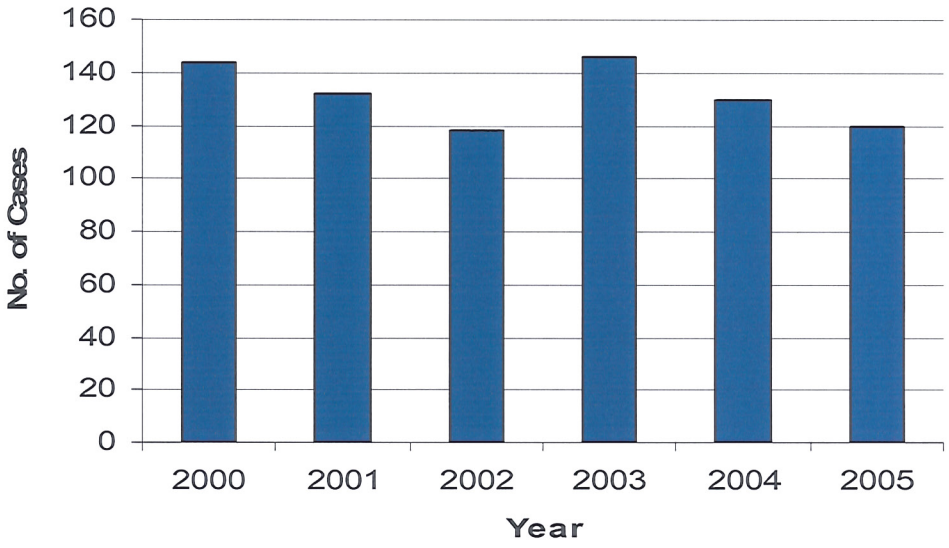
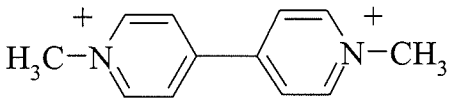


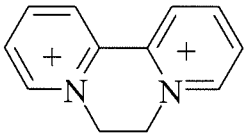
Figure 2-2 Numbers of paraquat poisoning cases in Malaysia from 2000 to 2005.

The QA group includes two non-selective herbicides namely paraquat and diquat and one highly selective herbicide, difenzoquat. The molecular structures of these compounds are shown in Figure 2-3.

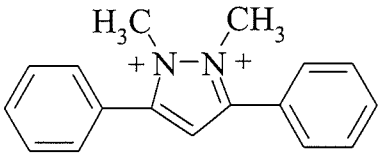
Paraquat (1,1'-dimethyl-4,4'-bipyridillium), also known as methyl viologen, was first synthesized in 1932 and was used as an oxidation-reduction indicator dye. In 1955, it was formulated as a dichloride salt and introduced into the market place as domestic and commercial herbicides [12]. Paraquat dichloride occurs at room temperature as an odourless and crystalline white powder with a relative molecular mass of 257.2. It is highly soluble in water (700 g/L at 20°C) but only slightly soluble in alcohol and insoluble in most other organic solvents. Its melting and boiling points are at 175-180°C and approximately 300°C, respectively.



Paraquat



Diquat



Difenzoquat

Figure 2-3 Molecular structures of paraquat, diquat and difenzoquat

Paraquat is a broad spectrum foliage-applied contact herbicide which enters the leaves and rapidly destroys plant tissues. Paraquat's mode of action depends on the photosynthesis process that occurs in the green parts of plants. This process will generate electrons that reduce the paraquat to form a relatively stable free radical. The bipyridinium free radical is rapidly oxidized in the presence of oxygen to produce superoxide that destroys membrane and cytoplasm and eventually leads to the frostbitten appearance of plants [15].

In man, when ingested in adequate dosage, paraquat has life-threatening effects on the lung, gastrointestinal tract, liver, kidney and other organs. Paraquat molecule will form a free radical by accepting an electron from cellular donors such as NADPH. In the presence of oxygen, paraquat radical donates its unpaired electron to generate superoxide and reform paraquat cation. This redox cycling continues to produce superoxide and further deplete NADPH. Excess amounts of superoxide lead to the production of hydrogen peroxide and hydroxyl radicals. Hydroxyl radicals are highly reactive and can cause lipid peroxidation and subsequently lead to tissue damage.

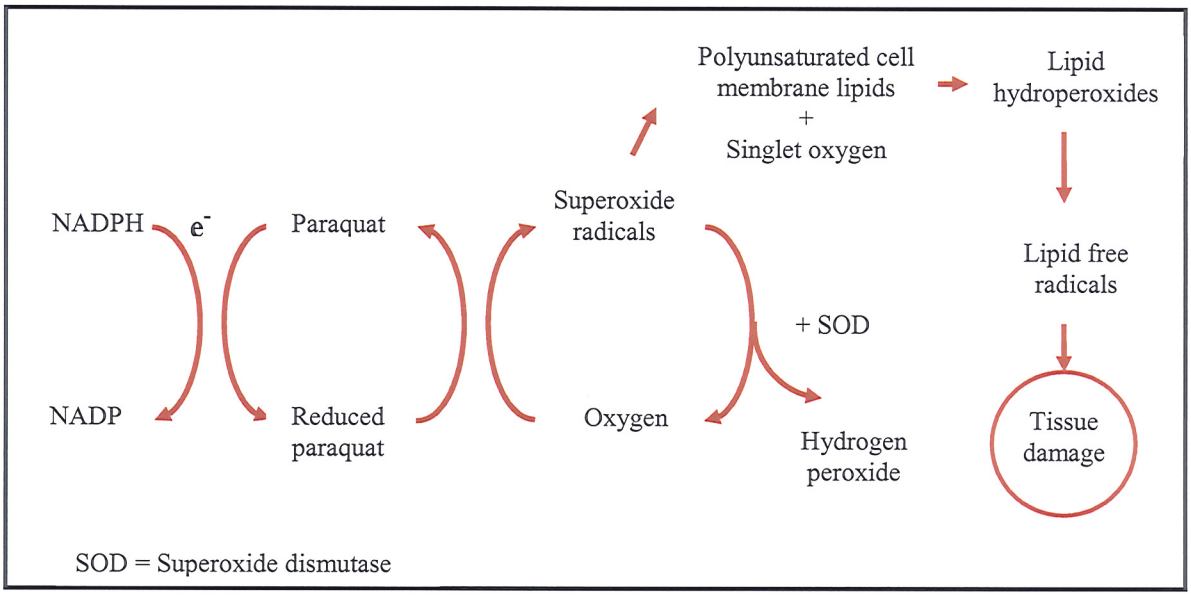


Figure 2-4 The mechanism of toxicity of paraquat.

Many fatalities have resulted from accidental or intentional ingestion. It is believed that an oral dose of only 1-2 g is fatal to most adults [16]. In fatal cases, post-mortem paraquat and diquat concentrations in blood 7 days after ingestion ranged from 0-0.44 mg/L and 0-0.06 mg/L, respectively [16]. The chances of recovery can often be predicted from the information of paraquat concentration in the plasma versus time of post ingestion as shown in Figure 2-5 [17]. The acute oral LD_{50} values for paraquat in rats and monkeys are about 120 and 50 mg/kg body weight, respectively.

In the United States of America, the Environmental Protection Agency (EPA) has classified paraquat among the products that are possibly carcinogenic to human beings. Paraquat is also believed to have deleterious effects on dopaminergic neurons and there is evidence suggesting that paraquat may be associated with the development of Parkinson's disease [18, 19].

To date, there is no antidote for paraquat poisoning but several methods have been used for handling paraquat toxicity [20]. Some use bentonite clay, Fuller's earth or activated carbon to prevent paraquat absorption in the gastrointestinal tract prior to gastric lavage. Absorbed paraquat is then removed from the bloodstream by hemoperfusion. Alternatively, an emetic is included in concentrated paraquat formulations to decrease paraquat absorption.

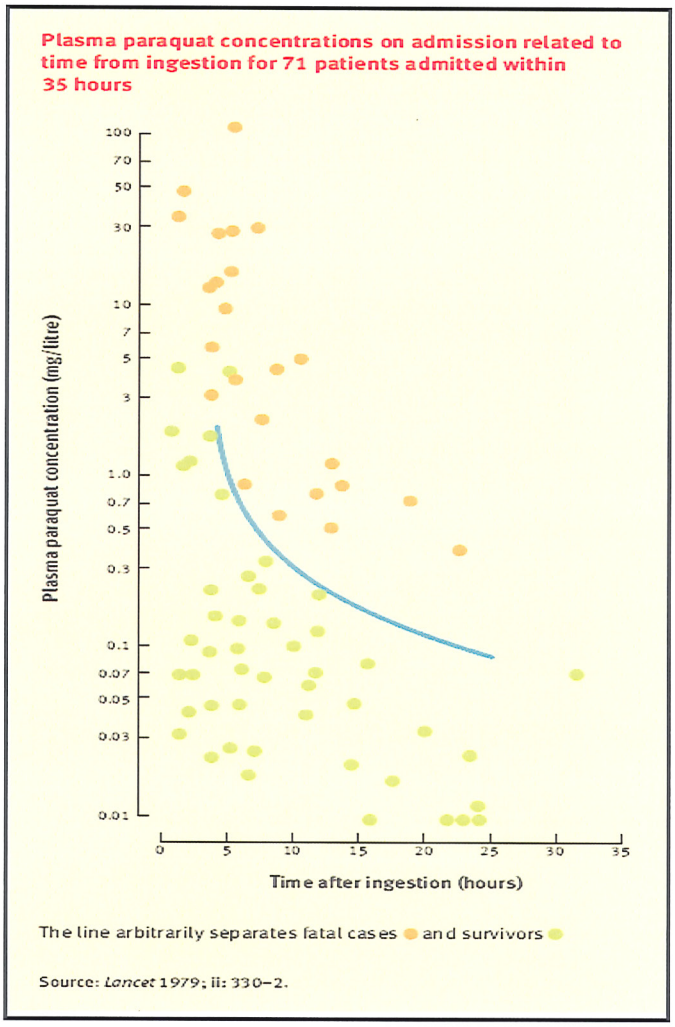


Figure 2-5 Plasma paraquat concentration vs time post paraquat ingestion.

Diquat (1,1'-ethylene-2,2'-bipyridillium) is a rapidly acting contact herbicide, especially against broad-leaved plants, which is particularly used as a desiccant. It is manufactured as dibromide salt and formulated with a surfactant wetting agent. Pure diquat dibromide monohydrate, with a relative molecular mass of 184.2 is an odourless, pale yellow crystalline powder and very soluble in water (700 g/L). It is slightly soluble in alcohol and insoluble in non-polar organic solvents. Its melting point is 180°C and boiling point is 300°C.

Diquat poisoning is less common than paraquat poisoning, therefore reports of human poisoning and animal experimental data for diquat are less extensive than for paraquat [21]. The acute toxicity (LD_{50}) value for diquat in rats is 400 mg/kg body weight. Diquat is observed to have a very low dermal absorption in man. However, when ingested, it has severe toxic effects on the central nervous system that are not typical of paraquat poisoning. The concentrations of paraquat in human tissue were higher than

for diquat, except in bile [22]. However, the distribution patterns of paraquat and diquat in human tissues are found to be quite similar.

Difenzoquat (1,2- dimethyl-3,-3,5-diphenyl-1H-pyrazolium) was introduced in 1975 as a selective, post-emergence herbicide normally used to control wild oats in small grain crops [23]. It is marketed in single formulation or tank-mixed with other herbicides such as bromoxynil, metsulfuron methyl and 2,4-D. Difenzoquat occurs at room temperature as a white to off-white odourless solid. It is soluble in water (765 g/L at 23°C) but poorly soluble in most organic solvents.

Difenzoquat has moderate toxicity by the oral route (LD_{50} for male rats is 270 mg/kg) and is slightly toxic via dermal exposure. Exposure to the skin in male rabbits exhibits an LD_{50} of 3540 mg/kg. It has been classified as a Group E carcinogen, a compound showing no evidence of carcinogenicity for humans. There are no literature reports of fatal human poisoning.

2.1.2 Quaternary Ammonium Drugs

QA drugs which are used as anticholinergics and muscle relaxants. In surgery, it is essential to relax muscles to prevent the patient from moving and enable the surgeon to reach the operation site. Muscle relaxant drugs act by interrupting neuromuscular transmission at the level of the nicotinic acetylcholine receptors at the motor end plate.

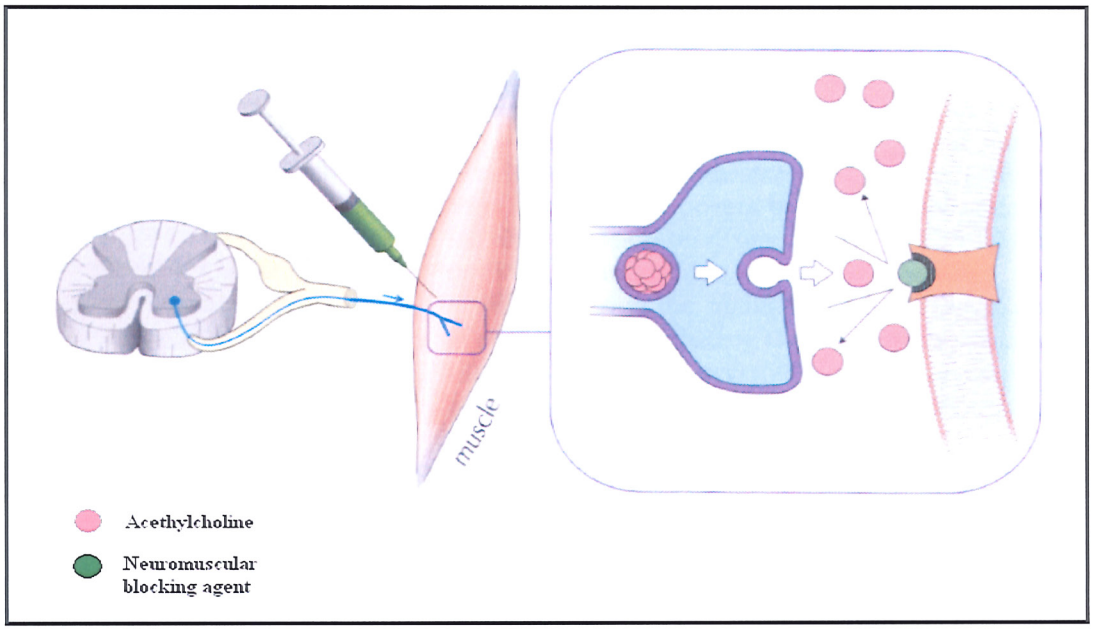


Figure 2-6 Peripheral-acting muscle relaxants affect receptors in the motor end-plate [24].

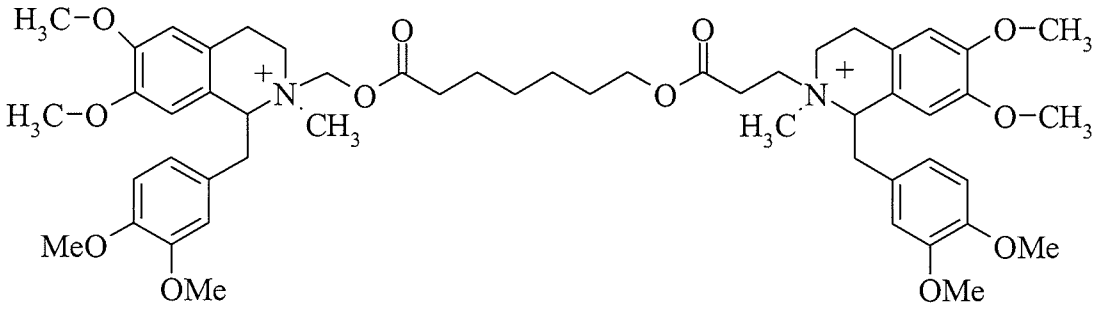
These drugs are known to be abused in cases of suicide. In homicide these drugs can be a potential poison because they are not routinely measured [25]. Although the numbers of human fatalities due to QA drugs are less than those caused by QA herbicides, this does not make the study of these drugs less important. These drugs are also known to be abused in equine sports and involved in accidental poisoning cases [26, 27].

Within the drugs used in the U.K., there are eight QA drugs that are the focus of this study, namely atracurium besylate, bretylium tosylate, edrophonium chloride, ipratropium bromide, mivacurium chloride, neostigmine, pancuronium bromide and rocuronium bromide. In Malaysia, all of these drugs except for bretylium tosylate and edrophonium chloride have been enlisted as prescription drugs under National Pharmaceutical Control Bureau. The molecular structures of these drugs are shown in Figure 2-7.

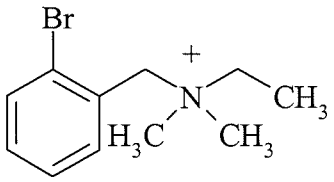
Atracurium (2,2'-[1,5-pentanediyl [bis-[oxy (3-oxo-3,1-propanediyl)]] bis[1-[(3,4-dimethoxy-phenyl)-methyl]-1,2,3,4-tetrahydro-6,7-dimethoxy-2-methylisoquinolinium] is a non-depolarising neuromuscular blocking agent of intermediate-duration used to produce skeletal muscle relaxation [28]. It is marketed under the trade name of Tracrium® as a solution and administered by intravenous injection as an adjunct to general anaesthesia.

It is miscible in water thus atracurium besylate injection is prepared in water to produce a clear or faint yellow solution. Overdosage of atracurium besylate will cause histamine release and therefore there is a possibility of life threatening anaphylactic reactions [29]. However, there is no significant acute toxicological data to be found in the literature.

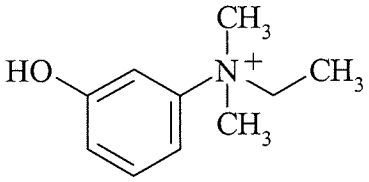
Bretylium tosylate (2-Bromo-N-ethyl-N,N-dimethylbenzenemethanaminium-4-methyl-benzene-sulfonate) is used in humans as antiadrenergic and antiarrythmic [30]. It is harmful if inhaled, swallowed or by skin contact and exposure can cause hypotension [31]. Its acute toxicity LD_{50} (intravenous) in rats is 17 mg/kg body mass. Following oral administration in humans, 70-80% of a bretylium dose is excreted unchanged in urine within 24 hours [31]. An additional 10% will be excreted within the next three days. Bretylium tosylate can be removed from the body by haemodialysis. There are few published case reports concerning human intoxication from bretylium tosylate [32].



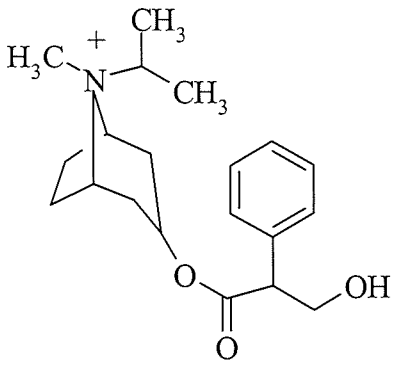
Atracurium



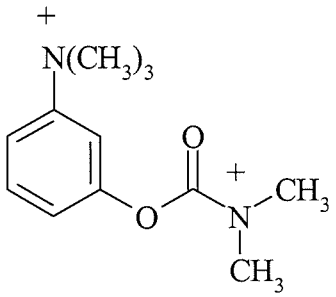
Bretylium



Edrophonium

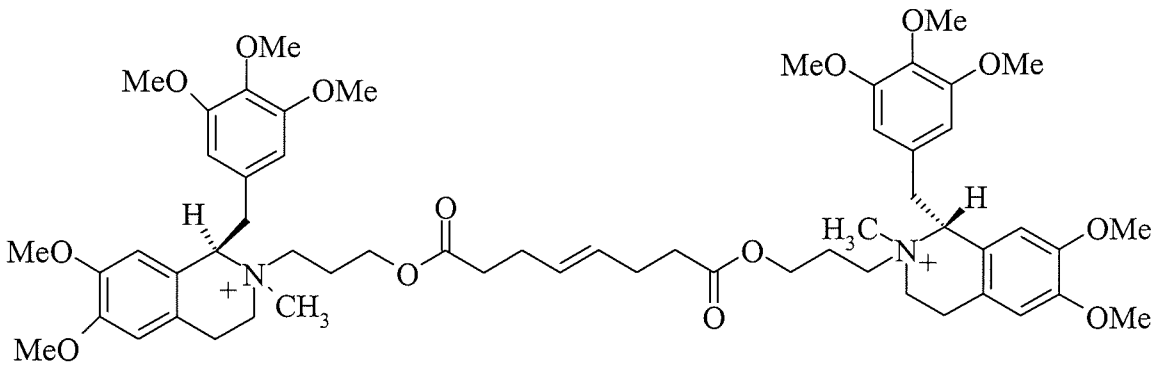


Ipratropium

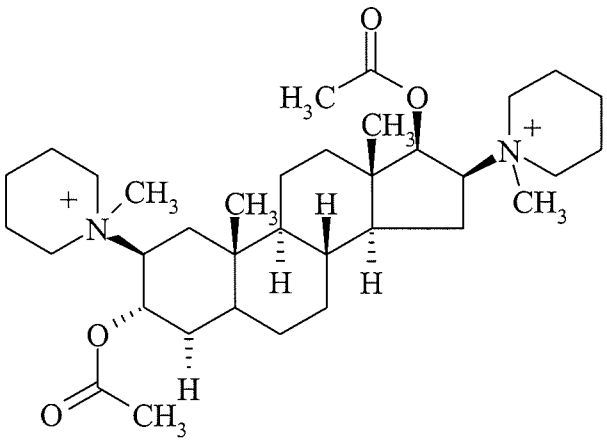


Neostigmine

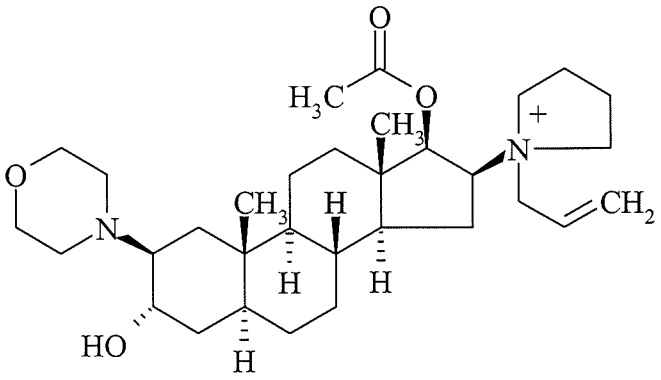
Figure 2-7 Molecular structures of quaternary ammonium drugs.



Mivacurium



Pancuronium



Rocuronium

Figure 2-7 (continued) Molecular structures of quaternary ammonium drugs.

Edrophonium chloride (*N*-Ethyl-3-hydroxy-*N, N*-dimethylbenzenaminium chloride) appears at room temperature as a white solid substance with a melting point ranging between 162°C and 163°C. It is used in humans to as a diagnostic aid for myasthenia gravis and oesophageal chest pain [33].

It may cause irritation when in contact with the skin and eyes. Exposure to edrophonium chloride may lead to cardiac and respiratory distress [34]. Since it is a cholinesterase inhibitor, it may cause abdominal cramps, diarrhoea, tight chest and miosis. Intravenous administration LD_{50} for mouse is 8500 μg/kg body mass. Toxicology properties in humans have not been thoroughly investigated.

Ipratropium bromide (1R,3r,5S,8r)-8 isopropyl-3-[(±) tropyloxyl]-tropanium bromide) is used in humans as a bronchodilator and antiarrythmic [30]. It appears as a solid white substance at room temperature with a melting point between 229-231 °C. Exposure to ipratropium bromide may cause dizziness, nausea and headache [35]. It may also lead to respiratory tract irritation, dry mouth and skin, changes in the heart rate and restlessness. The LD_{50} toxicity levels in rats for oral and intravenous administration are 1663 mg/kg and 15 700 μg/kg, respectively. Chronic exposure will cause reproductive hazards in both rats and rabbits.

Mivacurium chloride ((E)-(1R, 1'R)-2'-[4-octenedioyl-bis (oxytrimethyllene)][bis [1,2,3,4-tetrahydro-6,7-dimethoxy-2-methyl-1-(3,4,5-trimethoxy benzyl)-isoquinolium] dichloride) is administrated in human by intravenous injection as an adjunct to general anaesthesia to relax skeletal muscle and to facilitate tracheal intubations and mechanical ventilation [36]. It is prepared in water to give a clear pale yellow solution. No fertility studies have been performed on mivacurium; however, it has shown no adverse effects on foetal development in animal studies [37]. In the case of overdosing, it will cause prolonged muscle paralysis and decrease in blood pressure. Mivacurium is eliminated from the body through hydrolysis by liver esterase and excretion in bile and urine.

Neostigmine (3-[[(dimethylamino) carbonyl] oxy]-N,N,N-trimethylbenzenaminium methyl sulphate) is used in humans for the treatment of myasthenia gravis, the management of paralytic ileus and to combat non-depolarizing neuromuscular block induced by the muscle relaxants used in anaesthesia [38]. Neostigmine bromide and neostigmine methyl sulphate occur as odourless, bitter-tasting, white crystalline powders that are very soluble in water and soluble in alcohol. The melting point of

neostigmine methyl sulphate is from 144-149°C. Neostigmine oral administration and intravenous administration toxicity in rats is 51 and 165 mg/kg body mass respectively.

In humans, only 20% of the dose is excreted in urine following oral dosing and 5% of it is excreted as unchanged drug. Peak plasma concentrations following a 30 mg oral dose in man vary between 1 and 5 μg/L.

Pancuronium bromide (1,1'-[(3,17-bis acetyloxy) andros-tane-2,16-diyl] bis (1-methyl piperidinium) dibromide) occurs at room temperature as a white solid powder and soluble in water (0.1 g/mL) to form a colourless solution. It was introduced in 1968 and used in humans as a muscle relaxant under the trade name of Mioblock® or Pavulon® [13]. In about 30 states in the U.S., pancuronium is also used in conjunction with a typical anaesthetic, sodium pentothal, prior to a lethal dose of potassium chloride to execute death-row prisoners [39, 40]. Its acute toxicity LD_{50} (oral) and LD_{50} (intravenous) value for rats are 202 mg/kg body mass and 153 µg/kg body mass respectively. In man, its acute toxicity LD_{50} value is 143 µg/kg body mass. In a suicidal case presented by Klys *et al.*, a post-mortem blood pancuronium level of 1.1 mg/mL was observed [41]. It may cause irritation if in contact with the skin or eyes. Exposure to very small concentrations can cause a fall in blood pressure and increase in heart rate. Exposure to higher concentrations may lead to respiratory failure while chronic exposure can cause a mutagenic and reproductive hazard.

Rocuronium bromide (1-[2β, 3α, 5α, 16β, 17β]-17-(acetyloxy)-3-hydroxy-2-(4-morpholinyl)-androstan-16-yl)-1-(2-propenyl)pyrrolidinium) is supplied as a clear aqueous solution for intravenous injection. It is indicated as an adjunct to general anaesthesia to facilitate tracheal intubations and to provide skeletal muscle relaxation during surgery [42]. Rocuronium is excreted in urine and bile [43]. In man, 12-22% of a 0.6 mg/kg dose and 31% of a 1 mg/kg dose is excreted within 12 hours as the parent compound. No toxicological property data in the human is available but in the event of overdosage it will cause prolonged neuromuscular block.

2.2 Analysis Methods

Fast and reliable methods are desirable for the detection of herbicides and drugs in biological fluids due to their widespread application and also due to the many cases of injury and death caused by herbicide poisoning and drug abuse. Several quantitative

analytical methods for determining QA herbicides and drugs in biological fluids have been developed.

2.2.1 Spectrophotometric methods

A colourimetric measurement of paraquat and diquat has been described by Minakata *et al.* [44]. A red-coloured compound is produced on the reduction of diquat with 2-mercaptoethanol and a coloured paraquat radical is obtained by the reaction of paraquat and sodium dithionite. The maximum adsorption (λ_{max}) was achieved at 495 nm and 600 nm for diquat and paraquat, respectively. However, fading of the colour for reduced diquat of about 5% after 1 hour was observed.

The method has been improved for diquat determination by heating diquat in alkaline solution to produce a yellow-coloured derivative which is more stable and which has better absorptivity [45]. The maximum adsorption for diquat was achieved at 420 nm, with detection limits of 0.02 μg/ mL and 0.05 μg/ g for blood and liver, respectively. The colour was found to be stable for over 24 hours in cyclohexanol. These methods, however, require frequent preparation of the unstable reagents used. The use of flow-injection methods in adjunct to spectrofluorimetry for paraquat and diquat determination was also introduced to decrease analysis time and overcome the problems related to the unstable radicals produced in the process [46].

Fernandez *et al.* [47] reported the determination of cisatracurium and mivacurium in spiked serum and pharmaceuticals by spectrophotometry. The spectrophotometric method presented by Hassan [48] has provided simple and sensitive methods for the quality control analysis of ipratropium bromide in dose vials.

2.2.2 Chromatographic methods

In general, analytical methods based on chromatographic techniques are often preferred as they provide coverage for a very wide range of organic compounds. One of the earliest gas chromatography (GC) methods for the determination of paraquat in biological samples was described by Draffan *et al.* [49]. In this method, the reduction of paraquat salts with sodium borohydride has been employed to yield a hexahydro derivative which is amenable to solvent extraction and GC analysis. Determination of paraquat and diquat in blood and urine by GC has been described by Kawase *et al.* in

1984 [50]. Reinecke reagent was added to samples containing paraquat and diquat to produce precipitated Reineckate complexes. These complexes were then reduced by treatment with a mixture of sodium borohydride and nickel (II) chloride prior to analysis by gas-liquid chromatography (GLC).

There are very few papers published on the use of GC for the determination of QA drugs. Furuta *et al.* has described a lengthy pre-treatment process for selected QA drugs in biological fluids prior to GC analysis [51]. Limits of detection (LODs) ranging from 2 to 4 ng/mL were achieved. Due to the tedious pre-treatment process and the production of 'dangerous' derivatives during the process, liquid chromatography (LC) methods are favoured over GC.

LC analysis is preferred for more polar, less volatile and thermally labile compounds. A number of methods for the quantification of polar herbicides and drugs in biological fluids using high performance liquid chromatography (HPLC) have been developed. These methods showed excellent selectivity, reproducibility, speed and simplicity. Another distinct advantage is that only a small sample volume is required. An HPLC method for the determination of diquat metabolites in urine and serum has been published by Fuke *et al.* [52]. In this technique, urine and serum samples were first diluted with water and centrifuged. The supernatant was collected and injected into an HPLC system with a fluorescence detector. LODs of 0.1 ng/mL and 1 ng/ mL were achieved for diquat dipyridones and diquat monopyridones, respectively.

Rapid and simple analyses of paraquat in serum and plasma by HPLC with column switching have been described by Brunetto *et al.* and Lee *et al.* [53, 54]. The LOD achieved was 0.005 μg/mL by the first method and 0.1 μg/mL by the latter.

The determination of ipratropium bromide and related compounds by HPLC has been reported by Simms *et al.* [55]. The separations of those compounds were achieved using an acetonitrile/potassium phosphate buffer prior to UV analysis. The automated sample clean-up in combination with HPLC-UV method presented by Chiap *et al.* obtained a good recovery and repeatability for ipratropium bromide analysis in plasma samples [56].

Another HPLC-UV method for QA drugs has been reported by Varin *et al.* [57]. The method has been developed to determine neostigmine in human plasma and

cerebrospinal fluid and was used to study the pharmacokinetics of neostigmine in patients. Farenc *et al.* and Zecevic *et al.* reported HPLC methods for the determination of atracurium in plasma and pancuronium in drug formulations, respectively [58, 59].

Recently, remarkable developments have extended the applicability of HPLC analysis of QA compounds to more robust and efficient liquid chromatography/mass spectrometry (LC-MS) methods. LC-MS methods are very promising because mass spectrometric detection offers high sensitivity and a high degree of selectivity. LODs of LC-MS methods were found to be lower than HPLC methods and, due to the incorporation of solid-phase extraction (SPE) for sample pre-treatment, cleaner extracts have been achieved.

Lee *et al.* have reported the use of LC-MS-MS for the analysis of paraquat and diquat in human whole blood and urine samples [60]. The method is accurate, sensitive and recommended for use in clinical and forensic toxicology.

Unlike QA herbicides, more work has been reported on QA drugs in biological samples using LC-MS-MS. Farenc *et al.* and Cirimele *et al.* have developed LC-ESI-MS methods to analyse several QA drugs in blood and plasma samples [61, 62]. In both methods, samples were subjected to a dichloromethane liquid-liquid extraction prior to analysis. The procedures were found to be suitable for forensic and pharmacokinetic investigations in humans.

Yiu *et al.* have developed a method for the simultaneous screening of twenty QA drugs in equine urine by an LC-MS technique [63]. In this method the drugs were extracted from the samples by weak cation exchange SPE. Recently, Usui *et al.* reported an LC-ESI-MS method for the simultaneous determination of pancuronium, vecuronium and their related compounds in human serum [64]. The samples were also extracted using weak cation exchange SPE prior to analysis. The LODs achieved for pancuronium and vecuronium were 68.5 pg/mL and 50.3 pg/mL, respectively. This method is claimed to be helpful in the forensic and clinical fields.

2.3 Extraction Techniques for Analytical Toxicology

Apart from the identification and confirmation analyses, extraction procedures are also critical preparation processes in analytical methods. Sample preparation takes up two-

thirds of the analysis time in chromatographic analysis. Therefore, the improvement of these procedures is very important to reduce analysis time and produce more precise results. In toxicology sample analysis, SPE is a widely used technique which is able to isolate and preconcentrate the analytes prior to their separation and detection. SPE has gained acceptance among the analytical community and has gradually replaced traditional liquid-liquid extraction (LLE) as the SPE technique offers more advantages not only in the sense of performance but also in economic benefits.

There are wide ranges of sorbents with different kinds of physicochemical properties, pore sizes and particle sizes to ensure efficiency of extraction and quality of separation. So far, the most selective phases used for SPE are based on immunoaffinity [65, 66]. Although this type of SPE is found to be very sensitive and specific, it is less preferred due to its high cost. In addition, the operational conditions must be carefully controlled to avoid denaturation or mobilization of the antibodies.

Currently, researchers are working on the use of molecularly-imprinted polymers (MIPs) as an alternative technology to immunoaffinity. The findings have been very promising as MIPs show better selectivity and are highly stable, and rugged methods have been developed [67, 68, 69]. However, the use of SPE based on molecularly-imprinted polymers (MIP) to extract QA herbicides or drugs has not been done previously.

2.4 Aims

In a forensic toxicology investigation, biological fluids such as whole blood, plasma, serum and urine are most commonly analysed. However, screening of urine alone is insufficient to support toxicological findings due to factors such as the amount of excretion and the time lapse after the intake of the drug. Furthermore, in cases dealing with deteriorated blood, when total separation of plasma or serum from red blood cells is not possible, the sample of choice would be whole blood [70].

Therefore, the main aim of this project was to develop a reliable and sensitive method for the extraction and detection of QA herbicides and drugs in human whole blood. The identification of these compounds using the LC-MS-MS technique in combination with conventional SPE is developed and validated in real samples. An MIP material for

paraquat extraction was also subsequently prepared and investigated and this is reported in Chapter 9.

The theory of LC-MS techniques, SPE and MIPs is further discussed in the following chapters.

3 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY TECHNIQUE FOR ANALYSIS OF QUATERNARY AMMONIUM HERBICIDES AND DRUGS

3.1 Introduction

Liquid chromatography combined with mass spectrometry (LC-MS) is significantly more useful than gas chromatography - mass spectrometry (GC-MS) because it can in principle analyse a much larger number of organic compounds than GC-MS and it also eliminates the need for the time-consuming derivatisation steps often required for GC-MS. The vital part of this combined technique is the interface between the chromatograph and the mass spectrometer, which leads to the various types of LC-MS technique such as Electrospray Ionisation (ESI), Atmospheric Pressure Chemical Ionisation (APCI), Particle Beam, Thermospray and continuous flow Fast Atom Bombardment (FAB). For compounds which are highly polar and occur as ions in solution, the ESI interface is preferred to APCI [71]. Therefore, in this study, the method developed for QA compound screening and confirmation used LC-ESI-MS.

LC-MS is usually used to identify and quantify the substance or to confirm its molecular structure [72]. LC with tandem MS is often applied to enhance the selectivity of the complex-matrix sample by isolating a precursor ion prior to fragmentation steps, and therefore avoiding co-elution of analytes and interferences in samples [72, 73].

3.2 LC-ESI-MS Operation Principles

In work for this thesis, a Thermo Finnigan LCQ™ Deca XP instrument was used to develop the method (Figure 3-1). The instrument includes three major components, i.e. an HPLC system, an ESI interface and a mass spectrometer (MS) detector.

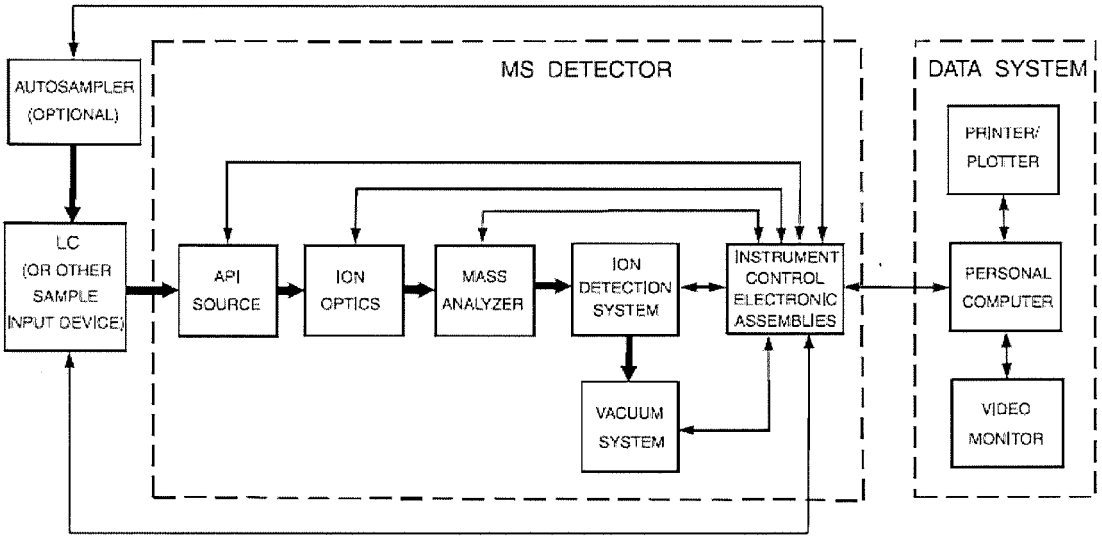


Figure 3-1 Functional block diagram of the LCQ Deca system [74].

3.3 Liquid Chromatography

Generally, LC is a technique for the separation of mixtures by differential migration through a column containing a micro particulate solid stationary phase. Reversed-phase liquid chromatography (RPLC) is the most important and widely used chromatographic technique in LC-MS. A mobile phase, that is either single solvent or mixed solvent, is degassed, filtered and blended prior to delivery onto the column by a constant flow pump. For high polarity ionic analytes such as QA compounds, mobile phases containing hydrophobic counter-ions should be used to increase the retardation of analytes in the column. The counter-ions must be sufficiently volatile to avoid deposition in the interface.

3.3.1 ESI interface

The LC eluent is introduced into an atmospheric-pressure ion-source region through a fused-silica inner capillary as shown in Figure 3-2. The capillary is inserted through the sample inlet and ESI needle.

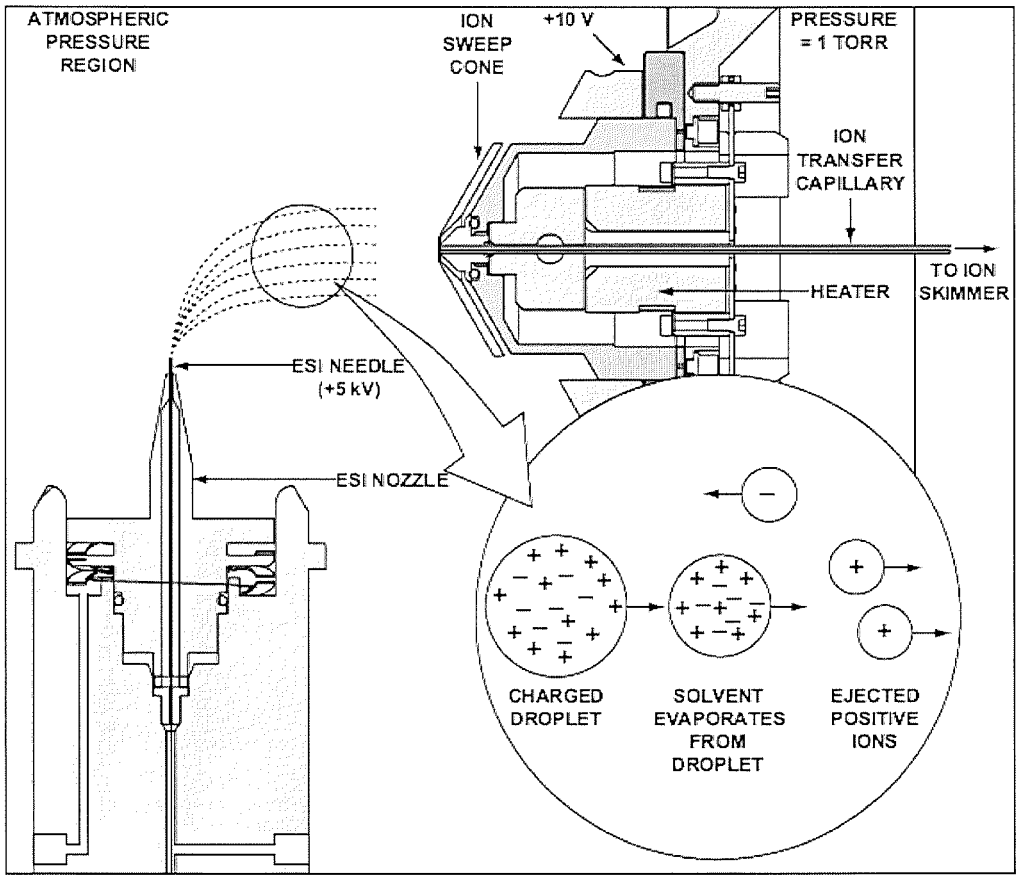


Figure 3-2 ESI process in the positive ion mode [74].

A large negative or positive voltage is applied to the ESI needle to break the emerging solvent from the needle into small charged droplets. These charged droplets will further disintegrate when the electrostatic repulsion is greater than the surface tension, leading to the formation of highly-charged microdroplets. Sample ions are transferred into the gas phase of the API stack (Figure 3-3) via an ion transfer capillary. The axis of the ESI probe is orthogonal to the axis of the ion transfer capillary, to keep the ion transfer capillary clean. In some cases, sheath gas and auxiliary gas consisting of 99% dry nitrogen are also directed at the droplets to assist in nebulizing the sample, stabilize the ion signal and help lower the humidity in the ion source. The charged molecules travelling from the ion transfer capillary will then enter the tube lens to be focused towards the opening of the skimmer. From the skimmer cone, the ions are directed into the ion optic assembly.

In order to optimize the sensitivity toward certain analyte, several MS detector parameters such as electrospray voltage, ion transfer capillary temperature, tube lens

offset voltage, capillary voltage and sheath and auxiliary gas flow need to be tuned. The settings of these parameters depend on the solvent flow rate and target analyte composition.

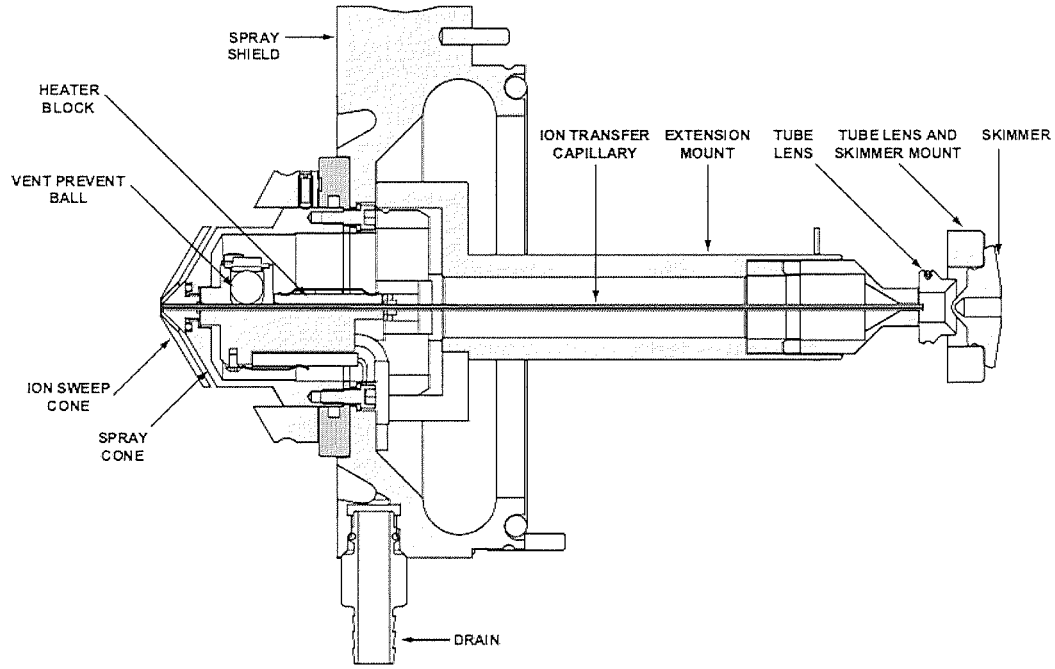


Figure 3-3 Cross sectional view of an API stack [74].

3.3.2 The Ion Optics

In the ion optic assembly, the ions will be separated according to their mass to charge (m/z) ratios (Figure 3-4). The Deca XP ion optic consists of a quadrupole and octapole which are separated by the inter-octapole lens. The quadrupole and octapole are arrays of rods that act as ion transmission devices. An RF and DC offset voltage are applied to the rods to give rise to electric fields that guide the ion along the axis of quadrupole/octapole and allow only ions of a certain m/z ratio to pass into the mass analyzer.

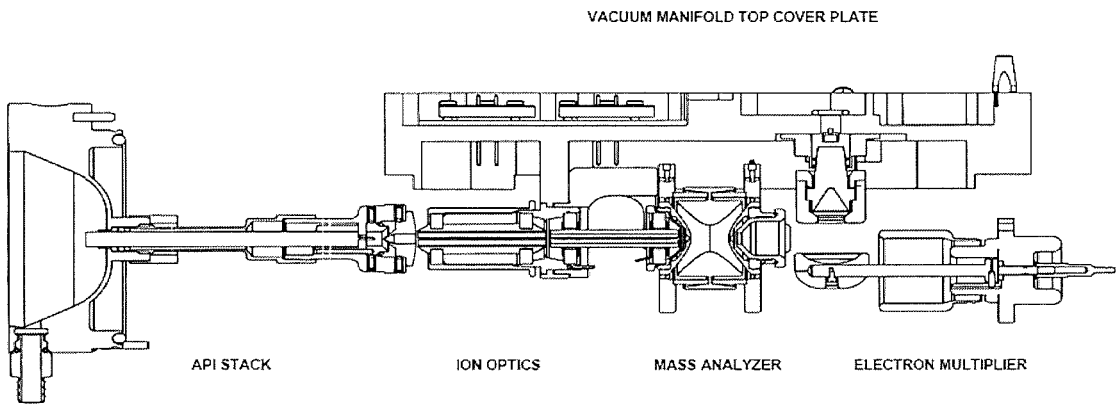


Figure 3-4 Cross sectional view of the LCQ Deca Plus ion optics [74].

3.3.3 Ion Trap Mass Analyzer

The ion trap mass analyzer consists of a ring electrode and a pair of end-cap electrodes (Figure 3-5). The end-cap electrodes sit above and below the ring electrode to form a hyperbolic cavity. The end-caps have small holes in the centre to permit the passage of ions into and out of the cavity. Ions which travel into the cavity will be trapped until an RF voltage is applied to the ring electrode. The trajectories of ions of particular m/z values then become unstable and the ions are ejected through the exit lens to the detector.

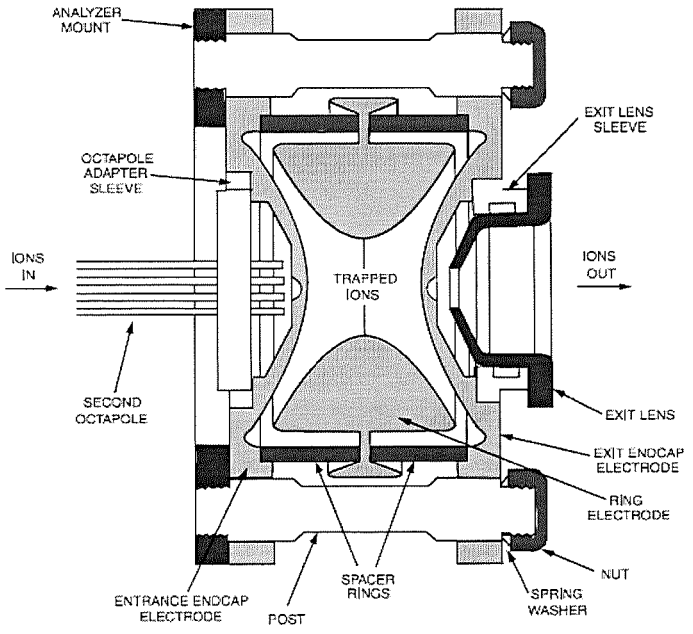


Figure 3-5 Cross sectional view of the mass analyser [74].

Once an ion reaches the detector, it will strike the surface of a conversion dynode to produce one or more secondary particles (Figure 3-6). The secondary particles are focused by the curved surface of the conversion dynode and are accelerated into the electron multiplier. If the secondary particles have sufficient energy, the electron multiplier cathode will eject electrons and these electrons will strike the inner surface of the cathode to produce more electrons. Thus, a cascade of electrons is created, resulting in a measurable current. The current is converted to a voltage by an electrometer circuit and recorded by the data system.

The main advantage of ion trap MS method is its ability to perform multiple stages of isolation and fragmentation of ions (MS^n) thus, easily enabled the identification of unknowns.

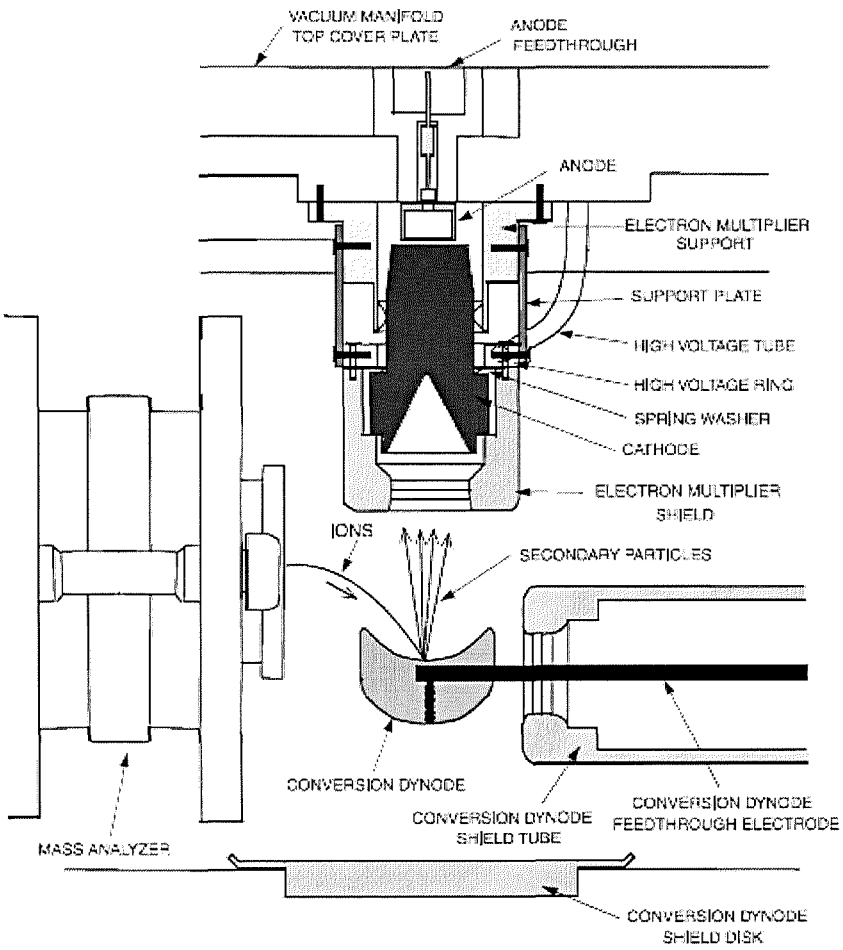


Figure 3-6 Cross sectional view of the ion detection system [74].

3.4 Method Development

3.4.1 Instrumentation

LC-MS-MS analyses of QA compounds was carried out using a Thermo Finnigan LCQ Deca XP instrument (Thermo Finnigan, San Jose CA, USA) equipped with a surveyor autosampler and MS pump system. Chromatographic separation was performed using an Atlantis® dC18 column (100 mm x 2.1 mm ID, 5 μm particle size) and guard column (2.0 mm x 2.1 mm ID, 5 μm particle size) from Waters, USA). Ionization of analytes was performed using the electrospray ionization (ESI) interface in the positive mode. An ion transfer capillary temperature of 275°C was employed. The nitrogen sheath and auxiliary gas flow rates were set at 15 and 5 arbitrary LCQ units respectively. QA compounds were analysed with MS parameters selected in Table 3-1 using product-ion scanning. The optimization of these parameters was explained in section 3.4.3 below.

3.4.2 Chemicals and Drug Standards

Paraquat dichloride, diquat dibromide and difenzoquat were obtained from Promochem (Middlesex, England). Atracurium, bretylium besylate, edrophonium, ipratropium bromide and pancuronium were purchased from Sigma (Poole, England). Neostigmine bromide was from Roche (England). Ethyl viologen, trifluoroacetic acid, pentafluoroacetic acid and heptafluorobutyric acid were obtained from Aldrich (Poole, England). Mivacurium chloride (Mivacron) was from Glaxo-Wellcome (Middlesex, UK). Rocuronium bromide (Esmeron) was from Ornagon (Oss, Holland). Benzyldimethylphenylammonium chloride monohydrate was obtained from Fisher (Loughborough, England). Ammonium acetate and ammonium formate were purchased from Fluka (Poole, England). Formic acid and methanol were of HPLC grade and were obtained from BDH Chemicals Ltd. (Poole, England).

3.4.3 Optimisation of MS Parameters

Before the LC conditions could be assessed, it was necessary to obtain good conditions for mass spectrometry. For this purpose, the HPLC system was by-passed using the by-pass valve facility fitted to the HPLC system and solutions of standards in methanol (1 μg/mL) were introduced into the mass spectrometer interface using a syringe pump at 5 μL/minute. The parameters which were investigated were electrospray voltage, ion

transfer capillary temperature, etc as described earlier. Optimum conditions were those which maximised the intensity of the molecular ion cluster and which produced ion fragments of sufficient intensity for ion reaction monitoring. QA compounds were analysed with MS parameters selected in Table 3-1 using product-ion scanning. The precursor ions used in each case were at m/z values corresponding to the molecular ion less one proton $(M-H)^+$. The fragmentation patterns of these compounds in LC-MS are rather different from the type of ion reaction found in GC-MS. Representative fragmentation reactions are illustrated in Figure 3-7 [75, 76].

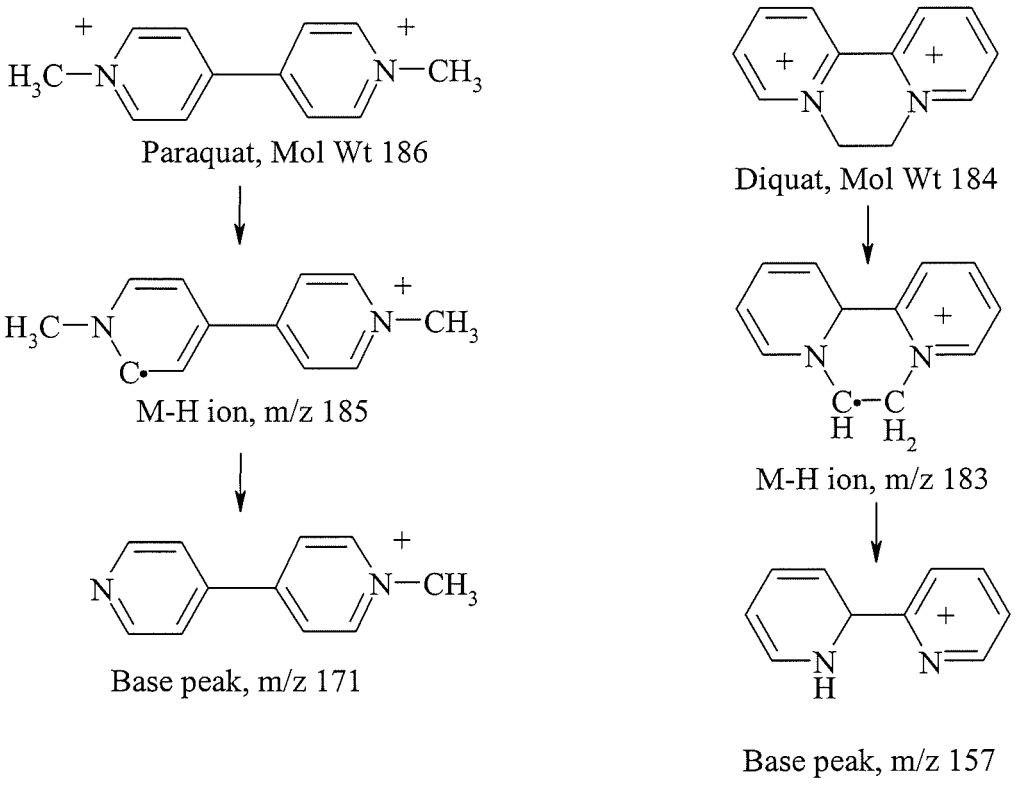


Figure 3-7 Formation of base peak product ions for paraquat and diquat.

Table 3-1 MS parameters for the analysis of QA compounds by LC-MS-MS in the positive ESI mode

QA compound	Precursor ion (m/z)	Product ions (m/z)	Relative Intensity	Collision energy (eV)
Atracurium	358	206.1	100.0	30
		207.1	8.40	
		327	5.30	
Bretylium	242	169.0	100.0	32
		71.9	20.2	
		224.9	17.8	
Diquat	183	157.1	100.0	39
		164.8	47.8	
		149.8	10.3	
Difenzoquat	249	208.1	100.0	47
		193.2	87.5	
		131.2	38.34	
Edrophonium	166	138.0	100.0	34
		137.0	32.3	
		148.8	12.7	
Ipratropium	332	166.1	100.0	35
		290.1	8.9	
		167.7	7.6	
Mivacurium	514	357.1	100.0	33
		428.2	68.3	
		342.3	44.5	
Neostigmine	223	208.0	100.0	40
		177.0	63.9	
		209.0	118	
Pancuronium	286	236.6	100.0	24
		100.0	9.3	
		206.8	12.3	
Paraquat	185	171.1	100.0	35
		166.8	52.3	
		166.0	12.3	
Rocuronium	529	487.2	100.0	32
		488.2	25.5	
		358.2	3.55	
Benzyldimethylphenyl-ammonium (Internal standard for drugs)	212	120.0	100.0	30
		121.1	40.2	
		134.0	37.4	
Ethyl Viologen (Internal standard for herbicides)	213	185.1	100.0	37
		195.0	86.5	
		194.2	3.2	

3.4.4 Investigation of Mobile Phase

In normal HPLC practice, acetonitrile is often used as an organic modifier due to its lower extracting power, thus ionic components like QA will be retained longer in the column and a better separation wil be obtained. However, methanol is preferred over acetonitrile to avoid deposition of a white film on the ESI source [77]. In order to increase the hydrophobicity of the QA compounds and so increase retention of QA, the addition of ion-pairing agents and acid buffer was investigated.

Three ion-pairing agents *viz* trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA) and heptafluorobutyric acid (HFBA), which possess different length of carbon chain, were added to the aqueous mobile phase component of the proposed gradient system (Figure 3-8).

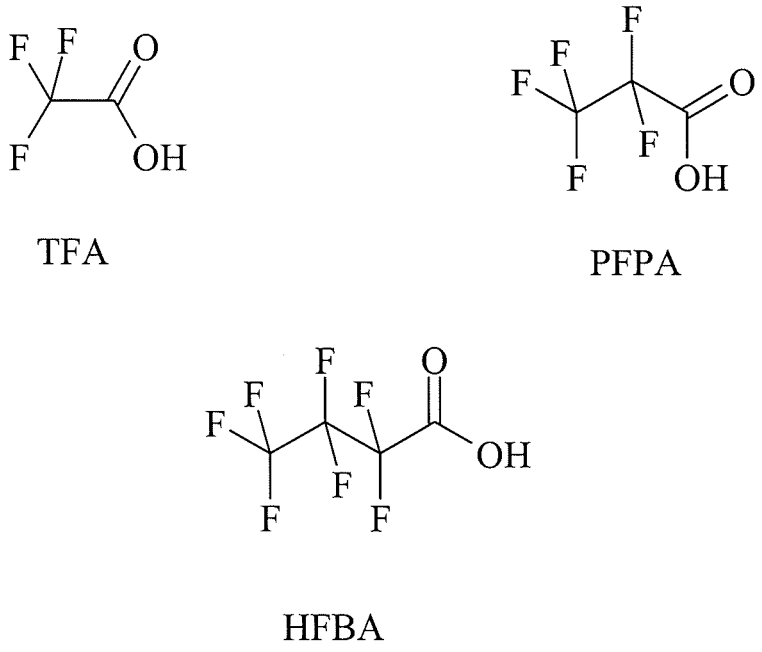


Figure 3-8 Molecular structures of TFA, PFPA and HFBA.

In this study, the QA compounds separation was carried out using 3 different mixtures of mobile phase solution as shown in Table 3-2 below.

Table 3-2 Gradient mobile phase components with different ion-pairing agents

Mobile Phase	Aqueous	Organic
A	10 mM TFA	Methanol
B	10 mM PFPA	Methanol
C	10 mM HFBA	Methanol

The concentration of ion pairing reagents in the aqueous solvent at 10 mM was chosen based on a previous study of QA herbicides in water samples by Castro *et al.* [78]. Furthermore a 10 mM concentration of ion pairing agent is within the limit suggested by the manufacturer [74].

Gradient elution was performed, starting at 95% aqueous solvent which was programmed linearly within 23 minutes down to a 60% aqueous solvent. In order to remove late eluting compounds, the solvent composition was switched to 5% organic solvent for 5 minutes. Subsequently, the system was programmed to regain its initial conditions over 2 minutes. The injection volume was 20 µl and the flow rate was set at 200 µl/min. The best mobile phase was selected by comparing the degree of separation between the compounds and response signal intensity.

Higher responses were obtained when using PFPA compared to TFA, especially for diquat. As shown in the chromatograms in Figure 3-9, the peak area obtained when using PFPA mobile phase is double the value obtained when using TFA.

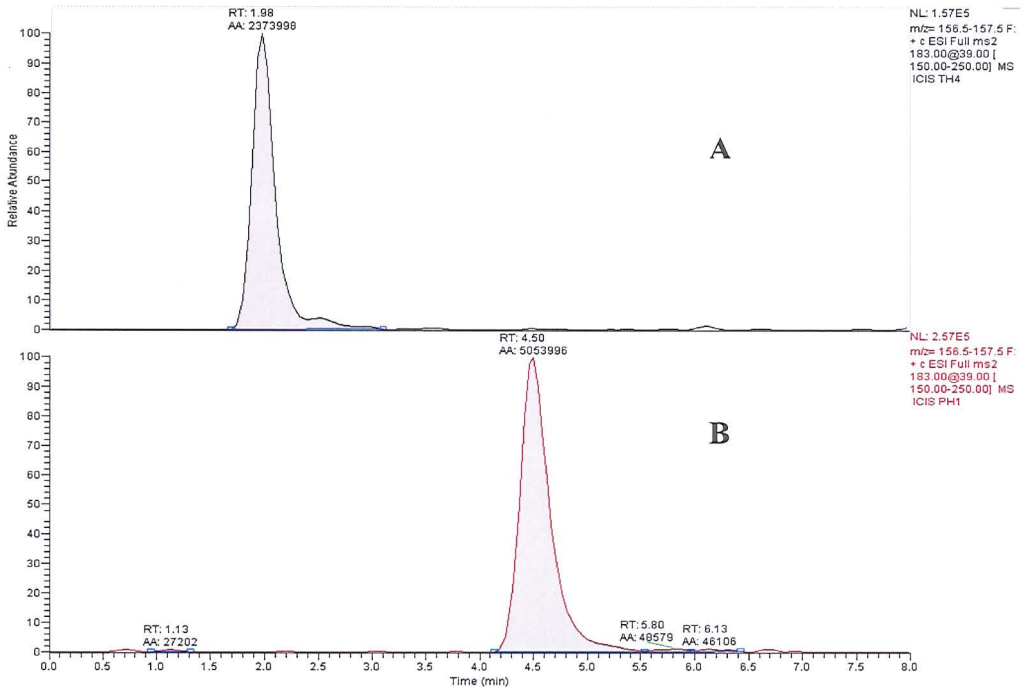


Figure 3-9 Chromatograms of the quantitation ion of diquat with A) 10 mM TFA and B) 10 mM PFPA as an ion-pairing agent. Conditions: Atlantis® dC18 column (100 mm x 2.1 mm ID, 5 µm particle size), gradient elution 95% aqueous solvent to 60% aqueous solvent in 23 minutes, methanol as organic component.

When using HFBA as an ion pairing agent, higher sensitivity was gained for all drugs compared to PFPA in the mobile phase. For pancuronium, broad peaks were observed when using TFA and PFPA, but a good peak was obtained at the end of analysis time with HFBA in the mobile phase. Figure 3-10 shows a comparison of chromatograms for neostigmine, bretylium, edrophonium and pancuronium obtained with PFPA and HFBA mobile phases. The peaks are annotated with peak areas and these can be compared directly. For convenience the areas are summarised in Table 3-3.

Table 3-3 Relative intensities of LC-MS peaks for QA drugs in mobile phases containing 10 mM PFPA and HFBA

	Relative Peak Areas*	
Drug	**PFPA**	**HFBA**
Neostigmine	**325**	**393**
Bretylium	**186**	**226**
Edrophonium	**260**	**357**
Pancuronium	**1**	**116**

* Pancuronium in PFP mobile phase set as 1

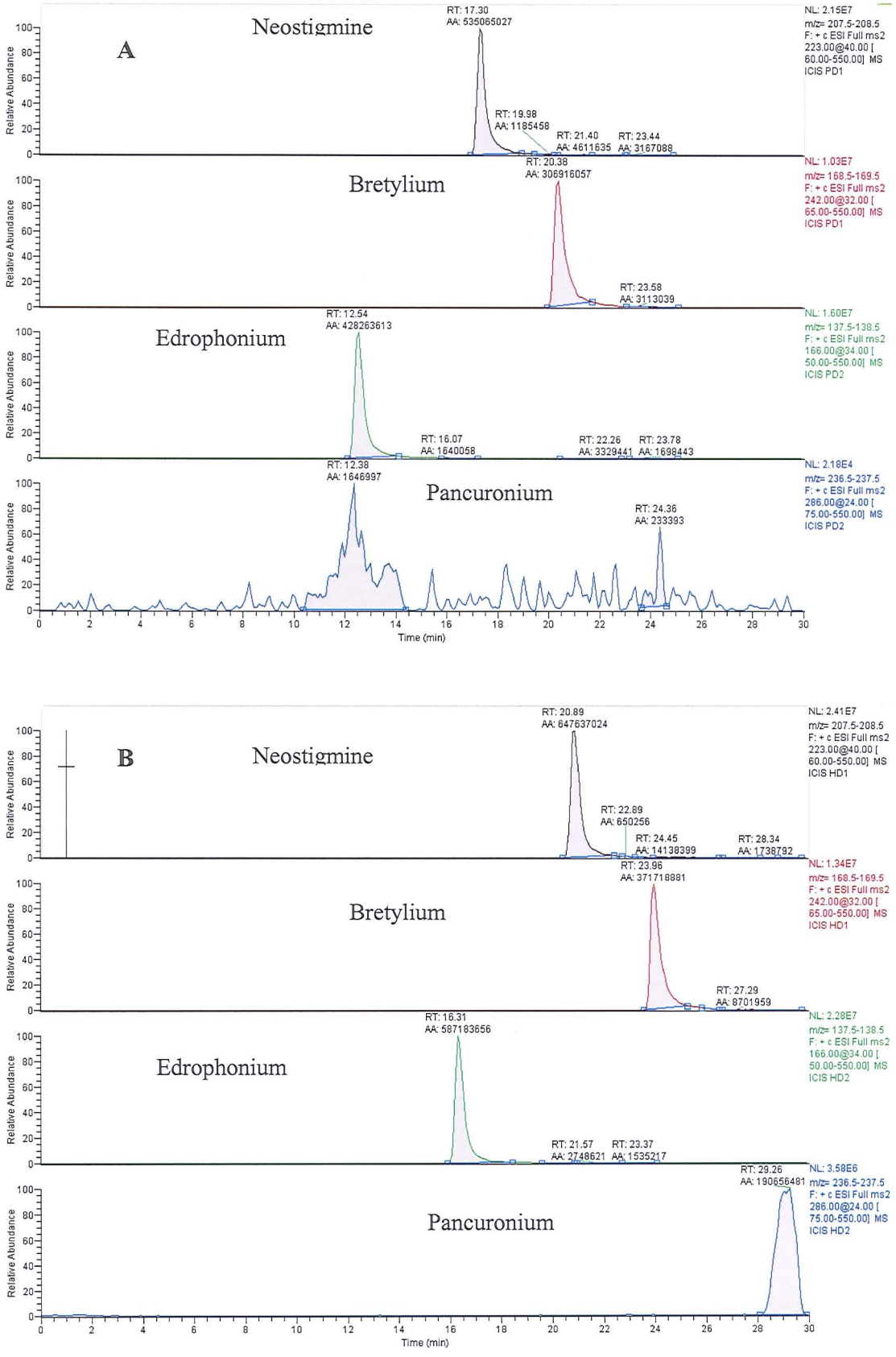


Figure 3-10 Chromatograms of the quantitation ions for selected QA drugs standard at a concentration of 100ng/mL with A) PFPA and B) HFBA as ion-pairing agent. Conditions: Atlantis® dC18 column (100 mm x 2.1 mm ID, 5 µm particle size), gradient elution 95% aqueous solvent to 60% aqueous solvent in 23 minutes, methanol as organic component.

3.4.5 Optimisation

From this initial study, it was obvious that better separations were obtained using the longer chain ion pairing agent. However, it also provided a longer retention time. In order to reduce the analysis time, the gradient programme was altered and the flow rate was also adjusted to obtain the shortest run time without having to sacrifice good separation between the compounds. For these trials, LC analysis was compared between 5 mM, 10 mM and 15 mM HFBA as solvent A and 100% methanol as solvent B. The elution program consisted of a linear gradient from 5 to 90% of solvent B within 18 minutes. The flow rate of 200 μL/ min was also compared to 250 μL/min.

Chromatograms of signals from paraquat ion in different concentrations of HFBA are shown in Figure 3-11. The highest concentration produced the best signal (Table 3-4). Although, higher concentrations of HFBA are not recommended in LC due to its corrosive properties, 15 mM of HFBA was used in the subsequent experiment. However, no significant changes in peak areas were observed between different mobile phase flow rates.

Table 3-4 Relative intensities of LC-MS peaks for paraquat in mobile phases containing different concentrations of HFBA

HFBA concentration	Relative Peak Area for Paraquat*
5 mM	**1.0**
10 mM	**1.2**
15 mM	**1.4**

*Peak area for paraquat in 5mM HFBA phase set as 1.0

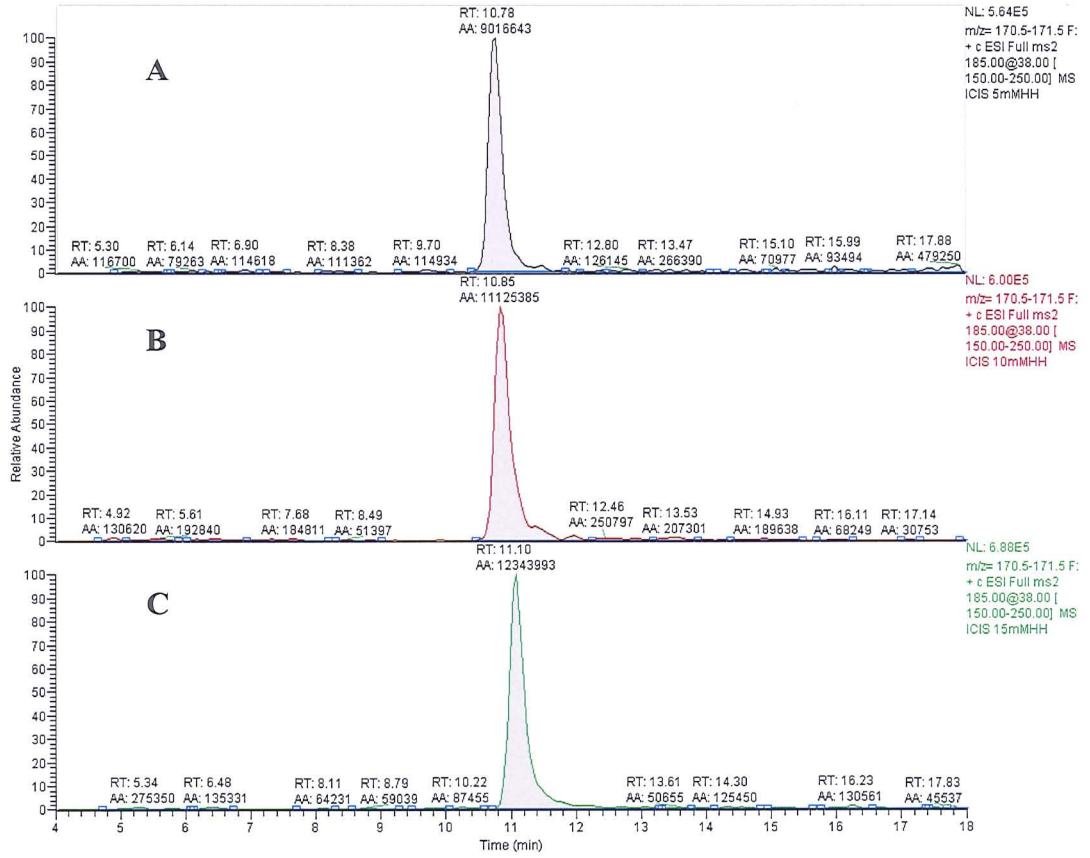


Figure 3-11 Chromatograms of quantitation ion of paraquat obtained with mobile phases containing different concentrationa of HFBA; A) 5 mM, B) 10 mM and C) 15 mM. Conditions: Atlantis® dC18 column (100 mm x 2.1 mm ID, 5 µm particle size), gradient elution 95% aqueous solvent to 10% aqueous solvent in 18 minutes, methanol as organic component.

3.4.6 Comparison of ammonium acetate and ammonium formate as mobile phase additives

The addition of ammonium acetate and ammonium formate to the mobile phase was investigated to assess if there would be any improvement in the ionisation of the QA compounds in the MS [79]. The experiments were carried out by adding 20 mM ammonium acetate or ammonium formate to the aqueous solvent component. The concentration of 20 mM was found to be the best by Lee *et al.* [54] and within the concentration limit recommended by manufacturer. Formic acid was added to adjust the pH to 3.30 to preserve the analytical column. The results (Figure 3-12 and Figure 3-13), clearly show using ammonium acetate or ammonium formate as an additives to QA drugs has increased its sensitivity to almost double compared to the ones without additive. However, no significant changes were found in sensitivity for QA herbicides except for paraquat; the response decreased when adding ammonium acetate.

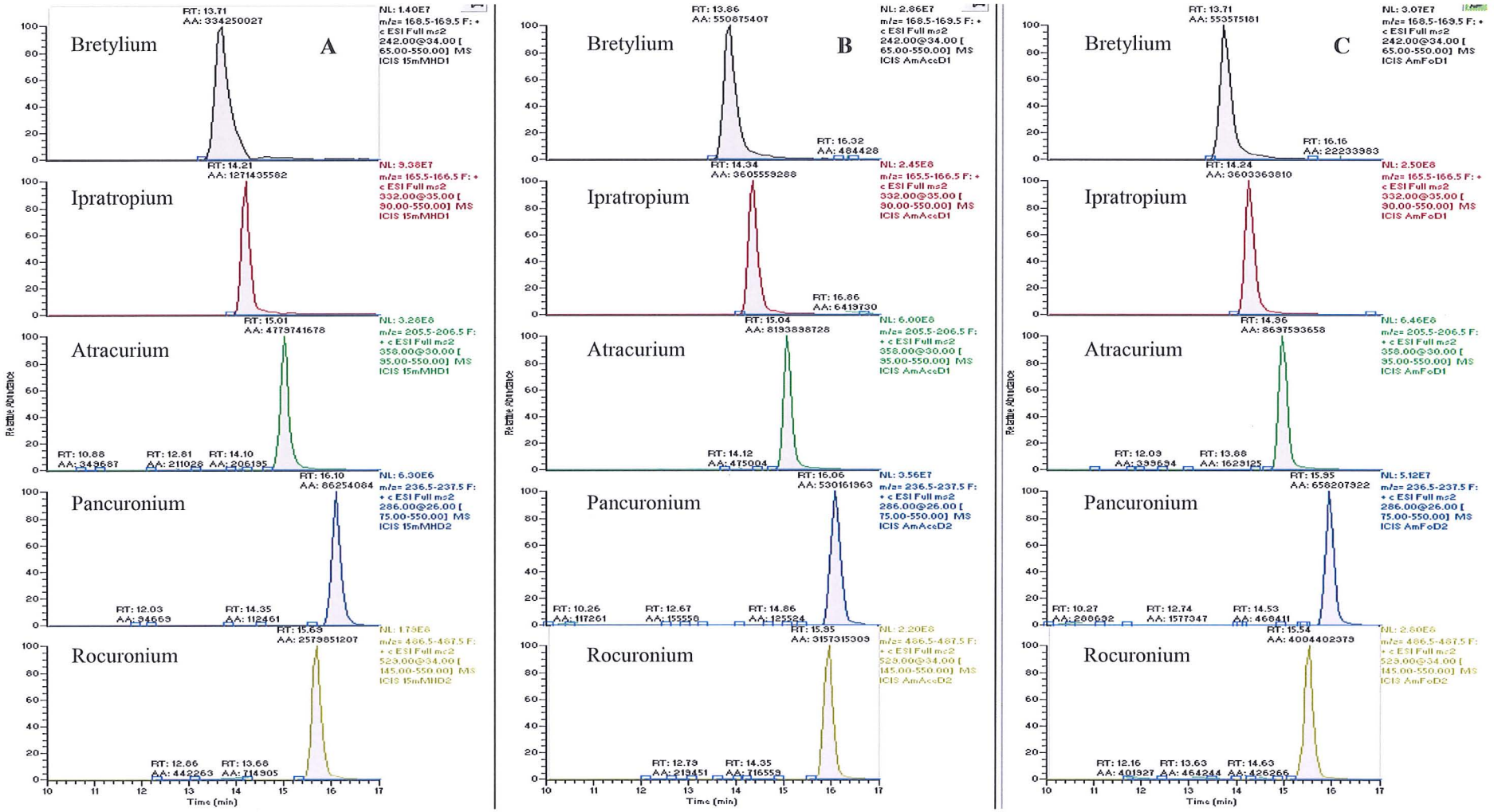


Figure 3-12 Chromatograms of quantitation ions for QA drug standards at the concentration of 100 ng/mL A) without additive and with B) 20 mM ammonium acetate and C) 20 mM ammonium formate in aqueous mobile phase solvent.

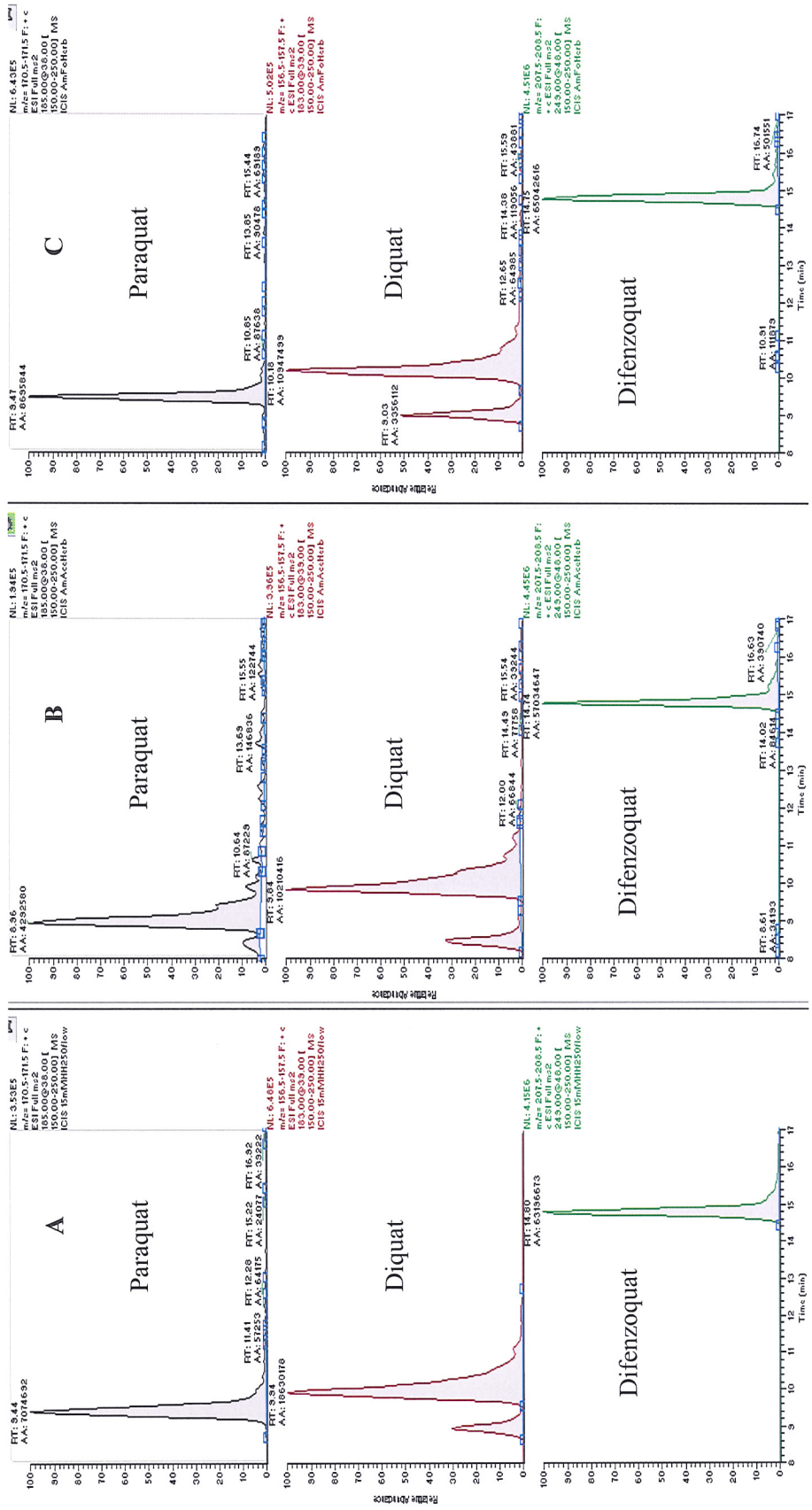


Figure 3-13 Chromatograms of quantitation ions for QA herbicide standards at the concentration of 100 ng/mL A) without additive and with B) 20 mM ammonium acetate and C) 20 mM ammonium formate in aqueous mobile phase solvent.

The peaks can be compared directly and are summarised in Table 3-5 and Table 3-6.

Table 3-5 Relative intensities of LC-MS peaks for QA drugs using mobile phases with different additives

Drug	Relative Peak Areas*		
	No additive	20 mM Ammonium Acetate	20 mM Ammonium Formate
Bretylium	3.9	6.4	6.4
Ipratropium	14.7	41.8	41.8
Atracurium	55.4	95.0	100.8
Pancuronium	1.0	6.1	7.6
Rocuronium	29.9	36.6	46.4

*Pancuronium in mobile phase with no additive set as 1.

Table 3-6 Relative intensities of LC-MS peaks for QA herbicides using mobile phases with different additives

Herbicides	Relative Peak Areas*		
	No additive	20 mM Ammonium Acetate	20 mM Ammonium Formate
Paraquat	1.0	0.6	1.2
Diquat	2.6	1.4	1.5
Difenzoquat	8.9	8.1	9.2

*Paraquat in mobile phase with no additive set as 1.

3.5 Discussion

All of the QA compounds which were used in this study were eluted from the Atlantis HPLC column within a reasonable time and with reasonable peak shapes despite the presence of the QA functional group. Optimised conditions for LC-MS-MS analysis of QA herbicides and drugs were developed which offered a practical analytical end step for a method to measure these substances in biological fluids. The best separation and most sensitive detection could be achieved by using a mobile phase consisting of 15

mM HFBA and 20 mM ammonium formate in aqueous solution and methanol as the organic solvent. The total elution time for all of the QA compounds analysed was within 16 minutes. These additive concentrations worked satisfactorily with the ESI interface to the mass spectrometer and did not result in blocking of the interface or cumulative build up of contamination in the source. HFBA contains a longer carbon chain than PFPA or TFA, and the organic content in the mobile phase therefore increased, resulting in a better retention and separation of the compounds. Addition of ammonium formate also aided in the response for all the QA compounds, presumably by means of a charge transfer process occurring in the ESI interface.

3.6 Conclusion

The optimised liquid chromatography-tandem mass spectrometry system for QA compound determination which was developed would be applicable for the intended purpose. This condition has been applied throughout the experiment for QA compounds analysis in the next chapter.

4 SOLID PHASE EXTRACTION METHOD FOR QUATERNARY AMMONIUM COMPOUNDS

4.1 Introduction

Solid Phase Extraction (SPE) is a rapid sample preparation method used to selectively extract, concentrate and purify target analytes prior to further analysis by chromatographic techniques. A typical SPE column is shown in Figure 4-1.

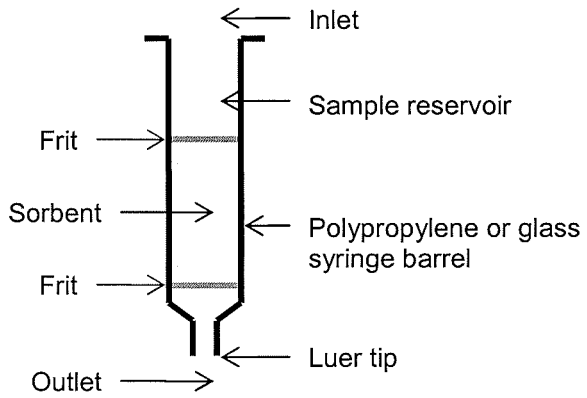


Figure 4-1 A typical SPE column.

Sample preparation with SPE consists of four basic steps: conditioning, sample loading, washing and elution. The schematic illustration of these steps is shown in Figure 4-2.

In the conditioning step, the SPE column is washed with organic solvent of intermediate polarity to prepare the sorbent for effective interaction with the analyte(s). In practice this means solvating the substituents on the surface of the silica which is the underlying support component of the sorbent (Figure 4-3). In the second step, the major aim is to ensure that the target analytes are quantitatively retained by the sorbent while the amount of bound impurities is minimised. The purpose of the wash step is to remove impurities that are bound to the sorbent without affecting the retention and recovery of the target analytes. In the final step, disruption of the analyte-sorbent interactions by using a "strong" solvent will elute target analytes. In the analysis of QA compounds these interactions include cation exchange and lipophilic interactions.

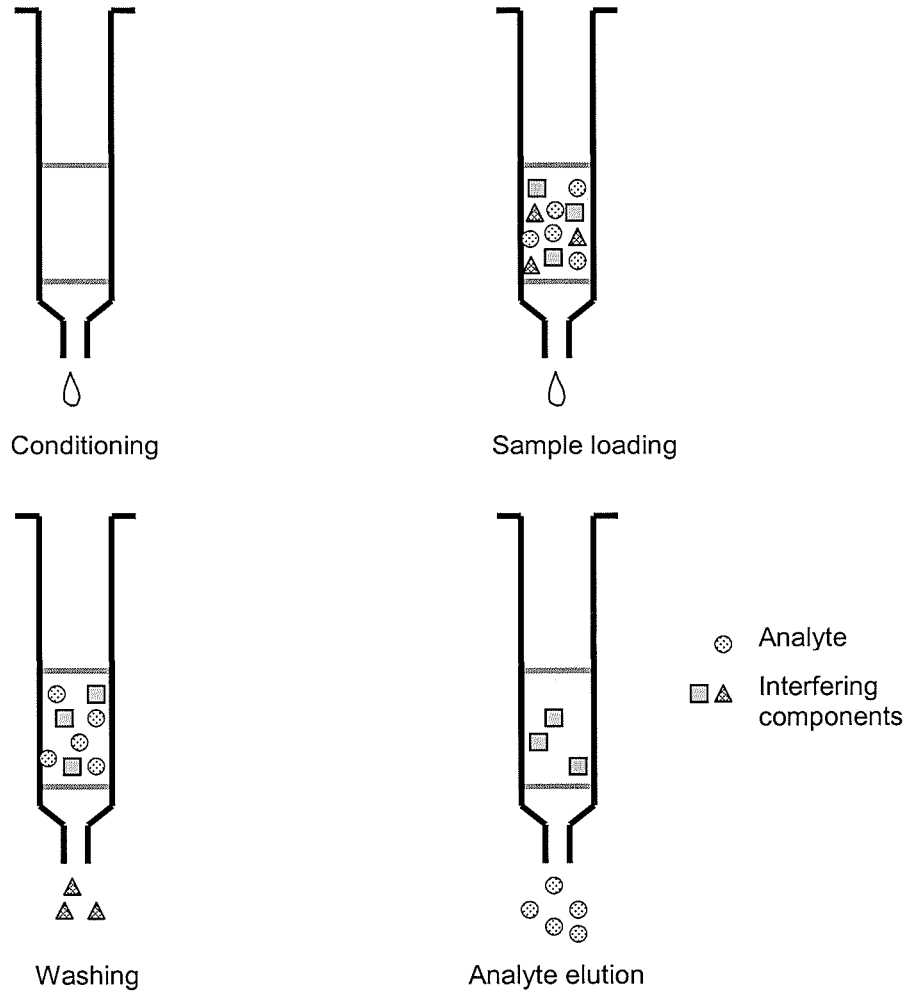


Figure 4-2 Schematic illustration of sample preparation using SPE.

QA compounds which are positively charged in solution may be best extracted using SPE with weak cation exchange material. The weak cation exchange SPE material contains an aliphatic carboxylic acid group that is bonded to the silica backbone (Figure 4-3).

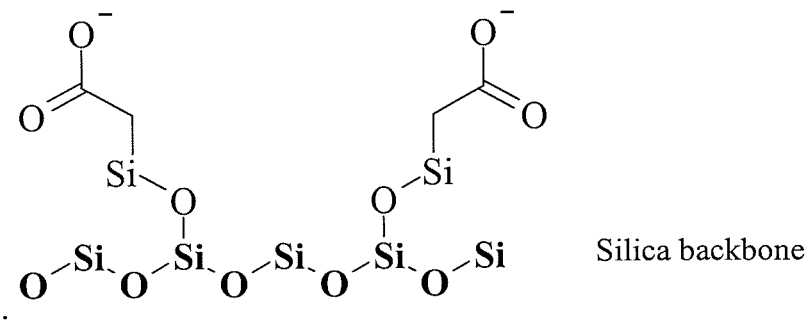


Figure 4-3 The structure of weak cation exchange SPE sorbent.

The carboxylic acid group has a pKa of approximately 4.8. Therefore, at 2 pH units above this value, 99.5% of the carboxylic acid groups will be negatively charged and will be able to retain the positively charged QA analytes. In order to elute the cationic analytes, the negatively charged carboxylic group must be neutralized by dropping the pH value at 2 units below its pKa. The other options are by i) neutralizing the charge on the analytes with a buffer, ii) using a high ionic strength buffer, iii) eluting with dilute acid or base in a solvent such as methanol or acetonitrile or iv) using a more selective counter-ion to displace the analytes. Option (i) is in practice not available for QA compounds which by their nature have a permanent positive charge.

In the work described in the following section, an optimised protocol for weak cation exchange SPE was developed, validated and applied to real toxicology case samples.

4.2 Method Development

4.2.1 Instrumentation

LC-MS-MS analyses of QA compounds were carried out using a Thermo Finnigan LCQ Deca XP (Thermo Finnigan, San Jose CA, USA) equipped with a Surveyor autosampler and MS pump system. Chromatographic separation was performed using an Atlantis® dC18 column (100 mm x 2.1 mm ID, 5 µm particle size from Waters (USA).

4.2.2 Chemicals and materials

Paraquat dichloride, diquat dibromide and difenzoquat were obtained from Promochem (Middlesex, England). Atracurium, bretylium besylate, edrophonium, ipratropium bromide and pancuronium were purchased from Sigma (Poole, England). Neostigmine bromide was from Roche (England). Ethyl viologen and heptafluorobutyric acid were obtained from Aldrich (Poole, England). Mivacurium chloride (Mivacron®) was from Glaxo-Wellcome (Middlesex, England). Rocuronium bromide (Esmeron®) was from Organon (Oss, Holland). Benzyldimethylphenylammonium chloride monohydrate was obtained from Fisher. Ammonium acetate and ammonium formate were purchased from Fluka (Poole, England). Formic acid and methanol were of the HPLC grade and were obtained from BDH Chemicals Ltd. (Poole, England). Bond Elut® LRC-CBA cartridges were purchased from Varian (CA, USA).

4.2.3 Standard solutions

Stock standard solutions (1 mg/mL) of QA compounds except for rocuronium bromide and mivacurium chloride were prepared by dissolving the dry chemical powder in methanol and were stored at 4°C in plastic bottles to avoid binding of QA to the surfaces of glassware. Stock solution is diluted to obtain a working standard solution of 1 μg mL. Rocuronium bromide and mivacurium chloride were prepared by diluting the formulations to 1 $\mu g\ mL^{-1}$ with methanol.

4.2.4 Blank Blood-Based Matrix

Blank human whole blood was not available for method validation. The closest alternative matrix was prepared as follows.

Time-expired packed red blood cells were obtained from the blood bank (Scottish Blood Transfusion Service, Glasgow Western Infirmary). These red blood cells were suspended in a ratio of 1:1 in isotonic saline (0.1 M sodium chloride solution) before use.

Ideally the best choice of sample matrix would be pooled whole blood although this would still be an approximation to autopsy blood. In this project, a suspension of red blood cells was used as an alternative to whole blood in method validation due to its availability. In post-mortem cases, the precision of quantitative measurements is lower than, for example, clinical cases due to autopsy blood being non-homogeneous and also because the degradation of the body has already affected the drug concentrations in blood due to post-mortem redistribution effects. Drugs from sites of high concentration in solid organs will diffuse into the blood, hence an artefactual elevation of drug levels in blood may be observed [70]. Therefore, no matter how well the analysis has been conducted, the data should be interpreted as approximations of the values which were present in life [80].

4.2.5 Solutions

4.2.5.1 Preparation of Mobile Phase

Mobile phase was prepared by adding 0.6306 g of ammonium formate and 0.975 mL of HFBA to 450 mL deionised water in a 500 mL volumetric flask. The volume was then made up to the mark with deionised water.

4.2.5.2 Preparation of pH 6.0 Phosphate Buffer

1.70 g of sodium hydrogen phosphate and 12.14 g of sodium dihyrogen phosphate were weighed into a 1 L volumetric flask, dissolved in water and the solution made up to volume with deionised water.

4.2.5.3 Preparation of pH 8.0 Ammonium Carbonate Buffer (0.02 mM)

0.94 g ammonium carbonate was weighed into a 500 mL volumetric flask. Approximately 450 mL of deionised water was added, then the pH was adjusted to 8.0 with potassium hydroxide and the solution was made up to volume with deionised water.

4.2.5.4 Preparation of pH 9.3 Ammonium Carbonate Buffer (0.01 mM)

0.47 g ammonium carbonate was weighed into a 500 mL volumetric flask. Approximately 450 mL of deionised water was added, the pH was adjusted to 9.3 with potassium hydroxide and the solution was made up to volume with deionised water.

4.2.6 LC-MS-MS Analysis

LC was carried out using HFBA (15 mM)-ammonium formate buffer (20 mM) adjusted to pH 3.30 with formic acid as solvent A, and 100% methanol as solvent B. The elution program consisted of a linear gradient from 5%-90% of solvent B within 18 min. 20 µl of sample was injected into the Atlantis® dC18 column, operated at 30°C and a flow rate of 0.2 mL/min.

Ionization of analytes was performed using electrospray ionization (ESI) in the positive mode. An ion transfer capillary temperature of 275°C was employed. The nitrogen sheath and auxiliary gas flow rates were set at 15 and 5 arbitrary LCQ units

respectively. QA compounds were analysed by product-ion scan MS-MS using the selected MS parameters listed in Table 3-1.

4.2.7 Optimization of extraction procedure

Initially, the SPE method was investigated using a protocol based on previously published work for QA drugs extraction by Yiu *et al.* [63]. The published method was applied to the extraction of equine urine samples using the conditions listed in Table 4-1.

Prior to the extraction steps, 1 mL blood sample was spiked with 50 ng standard and diluted with 4 mL ammonium carbonate buffer (pH 9.3). The mixture was vortex-mixed and centrifuged at 1121 x *g* for 10 minutes. After extraction, the eluate was evaporated to dryness with nitrogen stream and reconstituted with 100 μL initial LC mobile phase.

Table 4-1 SPE method used in initial investigation as published by Yiu *et al.* [63]

Step	Solvent
Conditioning	5 mL methanol, 5 mL water, 5 mL 001 M ammonium carbonate buffer (pH 9.3)
Washing	0.01 M ammonium carbonate buffer (pH 9.3), 5 mL methanol, 5 mL chloroform, 5 mL methanol
Elution	3 mL methanol containing 1% formic acid

Table 4-2 shows recoveries obtained for all selected QA drugs and herbicides except for mivacurium, rocuronium and difenzoquat. During the extraction, loading and washing steps, the liquids which are usually discarded were also collected and analysed by LC-MS. There were no analytes detected in either of residual liquids except that a high percentage of atracurium was detected in the wash liquid. However when the extraction was repeated without methanol in the washing solvent, the recovery of atracurium obtained after elution was 90.3%.

Table 4-2 Mean recoveries (%) of QA compounds using the initial extraction procedure

QA compounds	Mean recovery (%) ± SD*
Atracurium	< 10
Bretylium	87.9 ± 7.2
Diquat	25.4 ± 4.5
Edrophonium	91.3 ± 6.7
Ipratropium	92.6 ± 6.3
Neostigmine	92.8 ± 13.7
Paraquat	32.3 ± 7.4
Pancuronium	54.1 ± 8.2

*n=4

From the results, it was obvious that the procedure was not suitable for QA herbicides. Further investigation of the elution solvent was carried out for paraquat and diquat extraction. Three elution solvents were compared and the results are shown in Table 4-3. Higher percentage recoveries were obtained using 0.1 M HCl: methanol (30:70 v/v). However, to achieve the best recovery for both diquat and paraquat, a higher concentration of acid in the elution solvent was used *i.e.* 1.0 M HCl: methanol (30:70 v/v).

Table 4-3 Mean recoveries of diquat and paraquat using different elution solvents

Elution solvent (v/v)	Mean recovery (%) ± SD *	
	Diquat	**Paraquat**
1 % TFA: methanol (30/70)	18.8 ± 3.2	10.8 ± 4.6
1 % NH_4OH: methanol (30/70)	25.7 ± 2.2	15.4 ± 2.9
0.1 M HCl: methanol (30/70)	35.9 ± 2.8	22.1 ± 3.3
1.0 M HCl:methanol (30/70)	67.3 ± 1.4	46.8 ± 4.2

*n=4

The use of three different pH buffers *viz* pH 6.0 phosphate buffer and pH 8.0 and 9.3 ammonium buffers was also investigated. The results are shown in Table 4-4.

Table 4-4 Mean recoveries of QA compounds using eluting solutions with different pH values

QA compounds	Mean recovery (%) ± SD *		
	pH 6.0	pH 8.0	pH 9.3
Atracurium	88.4 ± 2.4	94.8 ± 1.2	94.3 ± 1.1
Bretylium	82.1 ± 5.3	92.7 ± 2.3	90.0 ± 2.9
Diquat	91.7 ± 3.9	73.1 ± 7.8	70.2 ± 8.2
Edrophonium	83.4 ± 10.1	92.0 ± 6.6	93.3 ± 6.7
Ipratropium	51.7 ± 8.3	96.0 ± 2.1	127.1 ± 3.4
Neostigmine	25.3 ± 5.6	94.0 ± 1.5	96.0 ± 1.2
Paraquat	83.5 ± 4.3	62.3 ± 11.7	50.9 ± 9.3
Pancuronium	79.3 ± 16.5	90.8 ± 6.2	89.4 ± 5.4

*n=4

4.2.8 Conclusion

The evaluation results showed that both QA drugs and herbicides require different pH and extraction conditions. For the analysis of QA herbicides, 1 mL blood sample was spiked with 50 ng standard and diluted 4 with mL phosphate buffer (pH 6.0). The blood samples were then vortex-mixed and centrifuged at 1121 x *g* for 10 minutes. Analysis of QA drugs were carried out in the same manner except that ammonium acetate solution (pH 8.0) was used as a buffer. The optimized extraction procedure is summarised in Table 4-5 below. The cartridge was eluted with 1 mL of 1.0 M HCl-methanol (70:30, v/v). The eluate was evaporated to dryness under nitrogen at 35°C and the residue was dissolved in 100 μL of the initial HPLC mobile phase.

Table 4-5 The optimized extraction procedures for QA drugs and herbicides

Step	Solvent	
	QA drugs	QA herbicides
Conditioning	3 mL methanol followed by 3 mL ammonium carbonate buffer (pH 8.0)	3 mL methanol followed by 3 mL phosphate buffer (pH 6.0)
Washing	2 x 3 mL of ammonium carbonate buffer (pH 8.0)	3 mL of phosphate buffer (pH 6.0) followed by 3 mL of methanol
Elution	1 mL of 1.0 M HCl-methanol (70:30, v/v)	1 mL of 1.0 M HCl-methanol (70:30, v/v)

4.3 Method Validation

4.3.1 Extraction method

The optimized procedure summarised in section 4.2.8 was used throughout the experimental work for method validation and application to real case samples.

4.3.2 Matrix Effect Study

This study was conducted to assess the effects of interferences which are co-extracted along with the analytes from the blood matrix during extraction on the LC-MS analysis of the extracts. Five replicates of 1 mL blank blood were spiked with 50 ng of QA compounds and another five replicates were prepared by spiking with the same amount of QA compounds but in 4 mL of loading buffer rather than blood. All of the samples were vortexed, centrifuged and extracted using the SPE procedure described earlier. 100 ng of internal standard was added to each replicate after the extraction.

The percentage matrix effect was calculated according to Equation 4-1, where 'a' and 'b' are the peak area ratios of the analytes to internal standard in neat solution and blood extract, respectively.

Equation 4-1 **Percentage of matrix effect = b/a x 100%**

A value of <100% indicates analyte signal suppression whereas >100% indicates analyte analyte signal enhancement due to the matrix interference. A summary of the observed matrix effects is given inTable 4-6.

From the data, it can be seen that blood matrix components co-extracted by SPE were found to cause both suppression and enhancement of the analytes during LC-MS, especially for edrophonium and paraquat. Therefore, all standard solutions were prepared in whole blood matrix to mimic the actual sample conditions.

Table 4-6 Blood matrix effect on sample extraction

QA compound	% Matrix effect*	RSD of Recovery (%)
Atracurium	105.9	15.6
Bretylium	91.1	7.3
Diquat	101.2	12.6
Difenzoquat	84.2	16.1
Edrophonium	65.0	15.5
Ipratropium	78.8	16.7
Mivacurium	105.9	14.4
Neostigmine	85.5	9.5
Pancuronium	112.7	15.9
Paraquat	144.7	16.3
Rocuronium	86.6	8.1

*n=4

4.3.3 Recovery Study

QA compounds were spiked at 50 and 100 ng/mL each in 1 mL aliquots of a drug free human blood sample (n=6), then were processed using the SPE procedure. 100 ng of internal standard (ethyl viologen for herbicides and benzyldimethylphenylammonium chloride monohydrate for drugs) was then added after the SPE. The recovery was determined by comparing peak area ratios obtained by dividing peak areas from product ion chromatograms of the QA compounds by the peak area of the internal standard versus the peak area ratios of the same concentration of pure standards and internal standard spiked in extracted blank blood matrix. Recoveries of QA compounds in human whole blood samples are presented in Table 4-7.

The recoveries of all QA compounds ranged between 79.7-105.1% and, apart from bretylium, RSDs were less than 10%. The average recoveries from blood were not significantly different according t values calculated using Student's t-test.

Table 4-7 Recoveries of QA compounds from human whole blood using the optimised method

	50 ng/mL		100ng/mL	
QA compound	**Mean recovery (%)***	**RSD of recovery (%)**	**Mean recovery (%)***	**RSD of recovery (%)**
Atracurium	105.1	3.8	94.3	3.1
Bretylium	79.7	17.5	90.7	15.7
Diquat	99.7	10.5	98.3	2.5
Difenzoquat	99.9	9.4	100.4	5.9
Edrophonium	95.2	3.1	90.2	6.6
Ipratropium	103.1	4.9	96.1	6.5
Mivacurium	93.1	8.1	92.0	3.8
Neostigmine	101.5	5.8	90.6	4.4
Pancuronium	94.3	7.6	96.5	2.1
Paraquat	84.9	2.8	89.7	5.7
Rocuronium	102.4	4.7	97.2	6.6

*n=6

4.3.4 Linearity and Determination of the LOD and LOQ

QA compounds were spiked into human whole blood to achieve concentrations of 2, 5, 10, 25, 50, 100 and 200 ng/mL and these were then extracted by SPE and analysed by LC-MS as before (n=3). Calibration curves were prepared by plotting peak area ratios of standards/internal standard (100 ng) against the spiked analyte concentrations. These were subjected to linear regression analysis. Limit of Detection (LOD) values were calculated using Equation 4-2 and Equation 4-3 [81], where Y_B is the intercept, S_B is the standard error of the regression line and m is the gradient.

Equation 4-2 $$Y_{LOD} = Y_B + 3S_B$$

Equation 4-3 $$LOD = (Y_{LOD}-Y_B)/m$$

Lower Limit of Quantification (LLOQ) values were calculated using the same method but using 10 times the standard error of the regression line (Equation 4-4 and Equation 4-5).

Equation 4-4 $Y_{LLOQ} = Y_B + 10S_B$

Equation 4-5 $LLOQ = (Y_{LLOQ}-Y_B)/m$

The regression analysis between peak area ratios of QA compounds over the internal standard versus concentration showed a good linearity in the range of 1-200 ng/mL. The correlation coefficients (r) for calibration curves were better than 0.99. LODs and LLOQs for all QA compounds are shown in Table 4-8.

Table 4-8 Correlation coefficients, Calibration curve coefficients, LOD and LLOQ for QA compounds in human whole blood obtained using the optimised method.

QA compound	r^2	b	a	LOD (ng/mL)	LLOQ (ng/mL)
Atracurium	0.9994	0.0385	-0.0691	5.8	19.3
Bretylium	0.9997	0.0020	-0.0004	4.1	13.7
Diquat	0.9985	0.0038	0.0092	5.0	16.7
Difenzoquat	0.9997	0.0186	-0.0012	4.1	13.7
Edrophonium	0.9989	0.0003	0.0006	10.8	35.9
Ipratropium	0.9994	0.0115	-0.0148	5.9	19.5
Mivacurium	0.9927	0.0008	-0.0063	20.4	68.0
Neostigmine	0.9998	0.0075	-0.0059	3.6	12.1
Pancuronium	0.9981	0.0079	-0.0325	10.4	34.5
Paraquat	0.9984	0.0046	-0.0140	11.1	36.9
Rocuronium	0.9996	0.0180	-0.0264	4.9	16.2

Linear calibration curve equation is $y = bx + a$, where x is the analyte concentration and y is the peak area ratio of analyte and internal standard. The calibration curves were determined over the range 1-200 ng/mL.

4.3.5 Intra- and Inter-day Precision

The intra- and inter-day precision were determined by analysing human whole blood samples spiked to concentrations of 10, 50 and 100 ng/mL with QA analytes six times in the same day and once a day during 6 successive days. Intra- and inter-day precision results showed that good precision could be obtained with the method described above. Relative standard deviations (RSDs) for intra- and inter-day precision ranged from 0.7% to 18.6% and are shown in Table 4-9. At the LLOQ level, 20% for precision and accuracy is acceptable [82].

Table 4-9 Intra- and inter-day precision for QA compounds in human blood samples obtained using the optimised method (n=6)

QA compound	Amount added (ng/mL)	Intra-day		Inter-day	
		Amount detected (ng/mL)	RSD (%)	Amount detected (ng/mL)	RSD (%)
Atracurium	10	10.4 ± 1.6	15.4	8.4 ± 1.5	17.5
	50	49.2 ± 3.6	7.2	47.6 ± 4.1	8.5
	100	96.3 ± 10.9	11.3	96.3 ± 7.2	7.5
Bretylium	10	10.4 ± 1.4	13.2	9.7 ± 1.3	13.8
	50	50.0 ± 4.6	9.1	47.7 ± 4.1	4.7
	100	100.0 ± 8.5	8.5	98.9 ± 6.0	6.1
Diquat	10	8.7 ± 1.6	17.9	10.7 ± 0.9	11.9
	50	46.0 ± 8.2	17.8	47.0 ± 0.3	0.7
	100	91.5 ± 5.5	6.0	96.0 ± 1.5	1.6
Difenzoquat	10	7.6 ± 1.1	14.3	10.7 ± 1.8	7.8
	50	47.1 ± 1.84	3.9	50.9 ± 2.4	4.8
	100	98.6 ± 3.1	3.1	102.2 ± 3.2	3.1
Edrophonium	10	10.1 ± 0.9	8.8	9.5 ± 1.4	14.2
	50	44.4 ± 4.8	10.7	51.1 ± 2.2	4.7
	100	94.2 ± 4.3	4.6	98.1 ± 5.3	5.4
Ipratropium	10	8.9 ± 0.7	8.2	9.4 ± 1.8	18.6
	50	47.7 ± 3.4	7.1	41.6 ± 2.9	6.9
	100	98.9 ± 7.1	7.2	98.2 ± 7.3	7.5
Mivacurium	10	10.1 ± 1.9	18.6	9.3 ± 1.7	18.4
	50	49.1 ± 4.4	9.0	48.6 ± 6.9	14.1
	100	101.8 ± 13.0	12.7	91.8 ± 11.7	12.8
Neostigmine	10	10.2 ± 1.3	12.8	9.7 ± 0.5	5.0
	50	49.9 ± 3.2	6.4	48.8 ± 2.8	5.7
	100	95.6 ± 5.6	5.9	96.6 ± 3.5	3.6
Pancuronium	10	8.5 ± 0.6	6.7	9.0 ± 1.3	14.3
	50	47.3 ± 0.94	2.0	49.0 ± 3.0	6.1
	100	94.5 ± 2.6	2.8	95.7 ± 7.5	7.8
Paraquat	10	10.1 ± 1.0	9.4	9.8 ± 1.5	15.2
	50	48.5 ± 4.8	9.8	49.9 ± 2.3	4.6
	100	103.6 ± 9.1	8.7	102.6 ± 3.3	3.2
Rocuronium	10	9.8 ± 1.3	13.4	10.0 ±.5	4.8
	50	49.7 ± 2.2	4.4	41.5 ± 2.8	6.7
	100	99.1 ± 3.5	3.6	99.5 ± 7.4	7.4

4.4 Application to Real Case Samples

4.4.1 Blood Sample

Blood samples were obtained from the Department of Forensic Medicine and Science, University of Glasgow. Results obtained were reported as part of the case investigations.

4.4.1.1 Case 1

This case involved a 66 year old man who died due to paraquat poisoning 4 days after suffering from poisoning despite active therapy. A few days before he died, the deceased brought home three mineral water bottles containing Dextreone weedkiller from his workplace and placed them in his grocery bag. A friend, who helped him with his groceries, put the bottles in the fridge, believing them to be blackcurrant juice. It is surmised that he subsequently drank some of the contents of these bottles. He was subsequently taken to the hospital emergency department but died 4 days later, despite active therapy. Post-mortem examination revealed jaundice, bilateral pleural effusions, marked pulmonary oedema and mottling of the kidney, indicative of death as the result of multiorgan failure. In addition there was an ulcerative oesophagitis and microscopic examination of the lung revealed changes in keeping with ingestion of paraquat and its toxic effects. A blood sample was taken and the proposed methodology was applied to screening and confirmation of paraquat level.

Quantification of paraquat was performed using a duplicate set of calibrators containing paraquat at concentrations of 0, 10, 25, 50, 100, 200 and 300 ng/mL, also containing 200 ng/mL ethyl viologen as an internal standard.

Figure 4-4 shows the extracted ion chromatogram and product ion spectrum of paraquat standard. Both the retention time and the product ion spectrum of paraquat in the sample matched well with those of the authentic standard. Paraquat was therefore positively identified at a concentration estimated at 0.64 mg/L. There was insufficient sample available to repeat the analysis to obtain a result within the calibration range.

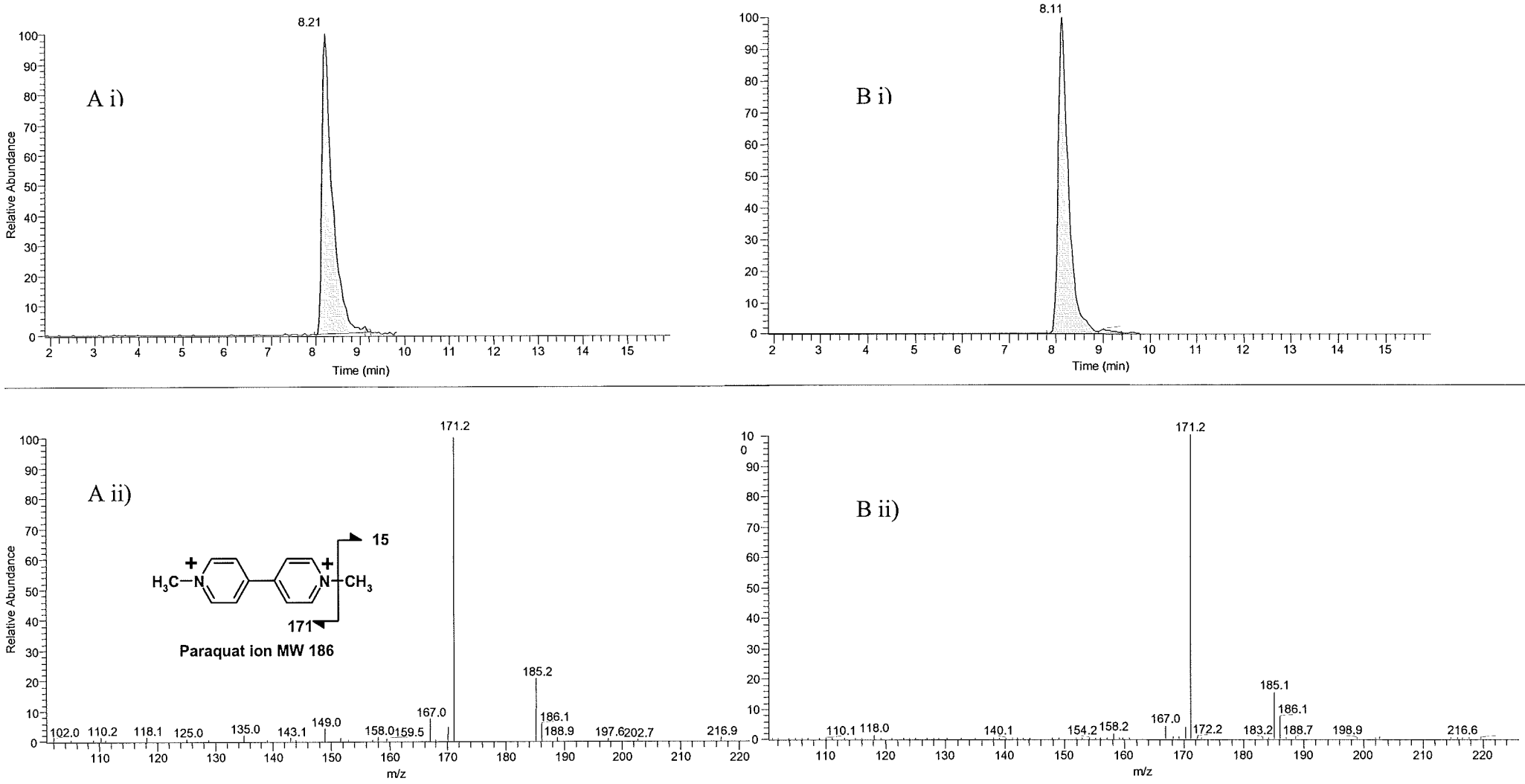


Figure 4-4 A i) extracted product ion chromatogram for m/z 171 and A ii) product ion scan of paraquat m/z 185 $[M-H]^+$ for the poisoning sample; B i) ion chromatogram of m/z 171 and B ii) product ion scan of paraquat m/z 185 $[M-H]^+$ of 300 ng/mL paraquat standard.

4.4.1.2 Case 2

A 64 year old man committed suicide by consuming approximately 200 mL of the weedkiller paraquat which he had kept in his garage for a number of years. He was taken to the hospital, but his condition deteriorated rapidly and he died the following morning. Post-mortem examination revealed superficial ulceration of the inside of his lower lip and reddening of the mucosa in the oesophagus. A blood sample was taken and the proposed methodology was applied to screening and confirmation of paraquat.

Quantification of paraquat was performed using a duplicate set of calibrators containing paraquat each at levels of 0, 10, 25, 50, 100, 200 and 300 ng/mL containing 200 ng/mL ethyl viologen as an internal standard. Figure 4-5 shows the extracted ion chromatograms and product ion spectra of paraquat in the standard and case sample respectively. This positive identification revealed the concentration of paraquat at 59 mg/L.

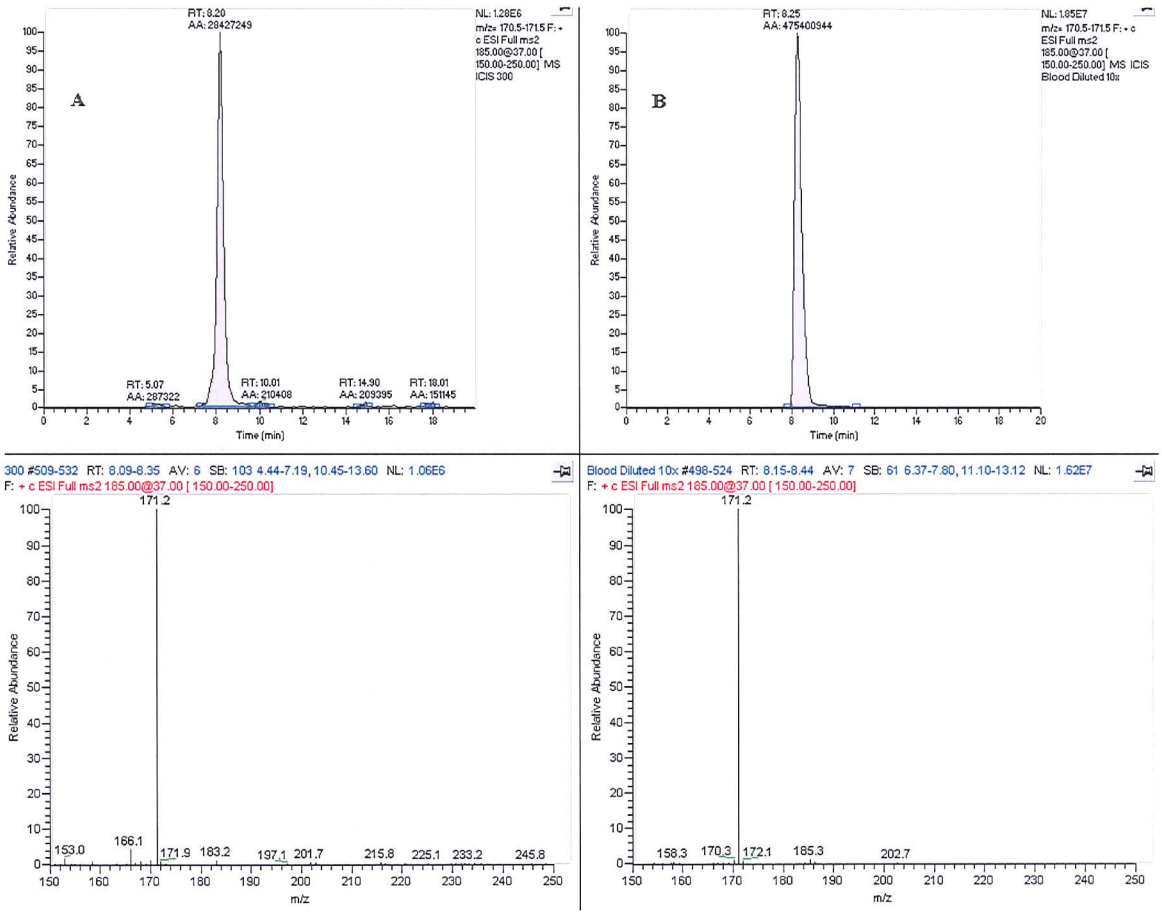


Figure 4-5 Ion chromatogram of m/z 171 and product ion scan of paraquat m/z 185 [M-H] of A) 200 ng/mL paraquat standard and B) for the poisoning blood sample.

4.4.2 Plasma Sample

A freeze-dried plasma sample was received from National Institute of Scientific Investigation (NISI), Seoul, Korea. The history of the sample is unknown but it was tested positive for paraquat in the NISI lab.

Prior to the analysis, recovery and linearity studies of QA herbicides in bovine plasma (Sigma, UK) were conducted using the optimised method for blood samples described in Sections 4.3.3 and 4.3.4. The recoveries, LOD and LLOQ values for QA herbicides in plasma are presented in Table 4-10.

Table 4-10 % Recoveries, % RSD, LOD and LLOQ values for QA herbicides in bovine plasma obtained using the optimised method for blood samples

QA herbicides	Mean Recovery (%) (50 ng amount added)	RSD of recovery (%)	LOD (ng/mL)	LLOQ (ng/mL)
Difenzoquat	31.5	6.3	29.8	49.6
Diquat	88.1	2.7	18.4	30.6
Paraquat	93.1	11.4	22.2	37.0

The results showed good recovery for paraquat and diquat. The LOD values for all herbicides were higher than obtained from blood samples, suggesting that the procedures used might not have the best conditions for plasma sample. The correlation coefficients for the calibration curves for all herbicides were better than 0.99, confirming a good linearity in the range of 5-300 ng/mL.

Quantification of paraquat in the plasma sample was performed using a duplicate set of calibrators containing paraquat at levels of 0, 10, 25, 50, 100, 200 and 300 ng/mL and containing 200 ng/mL ethyl viologen as an internal standard.

The concentration of paraquat in the sample was well outside the calibration range and too high to be estimated. There was insufficient sample available to repeat the analysis. The extracted ion chromatogram and product ion spectrum of paraquat standard in plasma are shown in Figure 4-6.

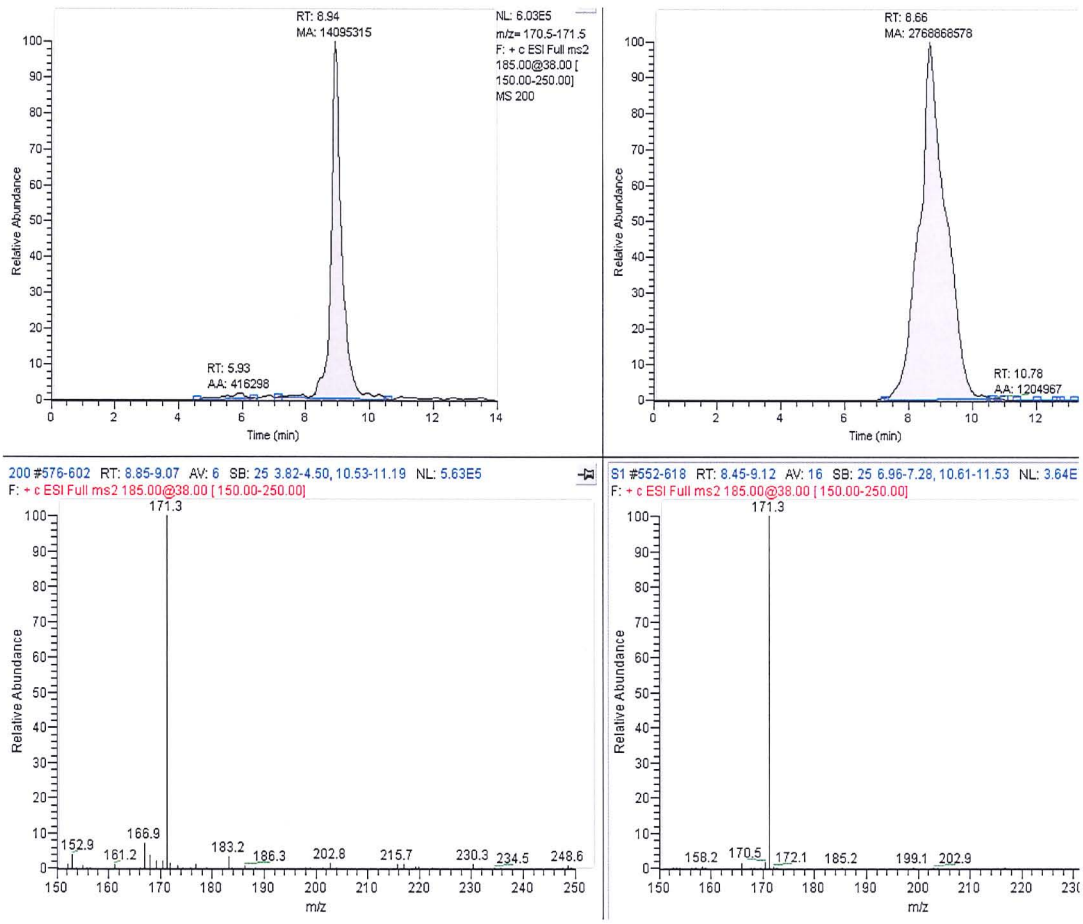


Figure 4-6 Ion chromatogram of m/z 171 and product ion scan of paraquat m/z 185 [M-H] of A) 200 ng/mL paraquat standard and B) for the poisoning plasma sample.

4.5 Discussion

The proposed solid-phase extraction procedure and LC-MS-MS method provide an accurate assay for the simultaneous determination of quaternary ammonium drugs and herbicides in human whole blood, Recovery and precision studies indicated that the method is sufficiently adequate for routine use in forensic toxicology.

The procedure has been successfully applied to a fatality following paraquat poisoning. The method also offers lower detection limit, better recovery and reproducibility when compared to previous methods [62, 63] for most QA drugs in blood samples. For the analysis of QA herbicides in blood, this method displayed a slightly better repeatability when compared to a study done by Lee *et al.* [60], and in addition produced data on difenzoquat.

However, the application of the method to the plasma sample gave less satisfactory LODs for all three herbicides investigated in this study. The chromatographic peak

shapes obtained in the analysis of plasma extracts were not as good as those for whole blood samples.

4.6 Conclusion

An SPE and LC-MS-MS method for QA compounds determination was validated. Results for QA analysis indicate that this method is accurate and sensitive enough to be used for forensic analysis in blood and plasma samples.

5 MOLECULARLY IMPRINTED POLYMERS

5.1 Introduction

Molecular imprinting is a method for creating polymeric material that displays selective molecular recognition characteristics towards the target molecules. The production of molecularly imprinted polymers (MIPs) involves the synthesis of highly cross-linked polymers in the presence of template molecules. Post-synthesis, the template is removed, leaving behind imprinted binding sites (cavities) within the polymer network that are complementary in size, shape and chemical functionality to the template. These binding sites are able to rebind with the template molecule, or other molecules that have close structural similarity to the template molecule, in a strong and selective manner. Figure 5-1 shows a schematic diagram of MIP preparation steps.

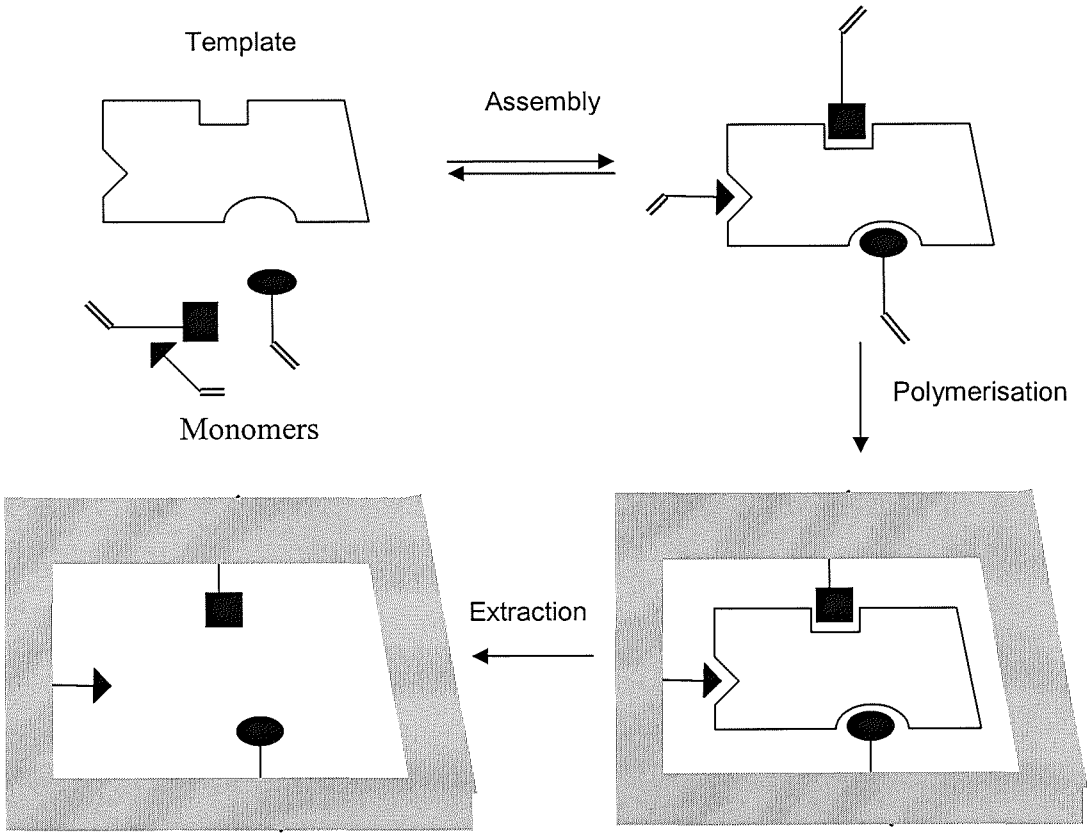


Figure 5-1 Schematic diagram of MIP preparation.

5.2 The Development of Molecular Imprinting

In the beginning of the 1930s, Polyakov *et al.* investigated the use of silica-based materials in chromatography [83]. In their approach, the silica framework was formed by mixing sodium silicate, water and a gelating agent, ammonium carbonate. Subsequently, the additives or a template (benzene, toluene or xylene) were added during drying. Unfortunately, the effort of this group was left unnoticed. In 1949, Dickey established another method for preparing molecular imprinting in silica matrices [84, 85]. The method used a slightly different approach compared to Polyakov's work, in which the template was added in the sodium silicate pre-polymerisation mixture prior to drying process. This technique has contributed to the present molecularly imprinted polymer (MIP) development methodology. However, after 15 years, the interest in molecular imprinted silica has declined due to the limitations in its stability and reproducibility. The extraction of the template/target molecule must be carried out carefully in order to retain the selectivity towards the target molecule. Moreover, the absorption capacities between batches were reported to vary by 30% [84].

The foundations for current molecular imprinting in synthetic organic polymer techniques were based on the pioneering work of the groups of Klotz and Wulff on covalent approaches [84,86]. Until the early 1980s Mosbach *et al.* introduced a non-covalent approach, which has been widely applied in modern imprinting procedures [87]. The number of publications on MIPs over the last 70 years is depicted in Figure 5-2.

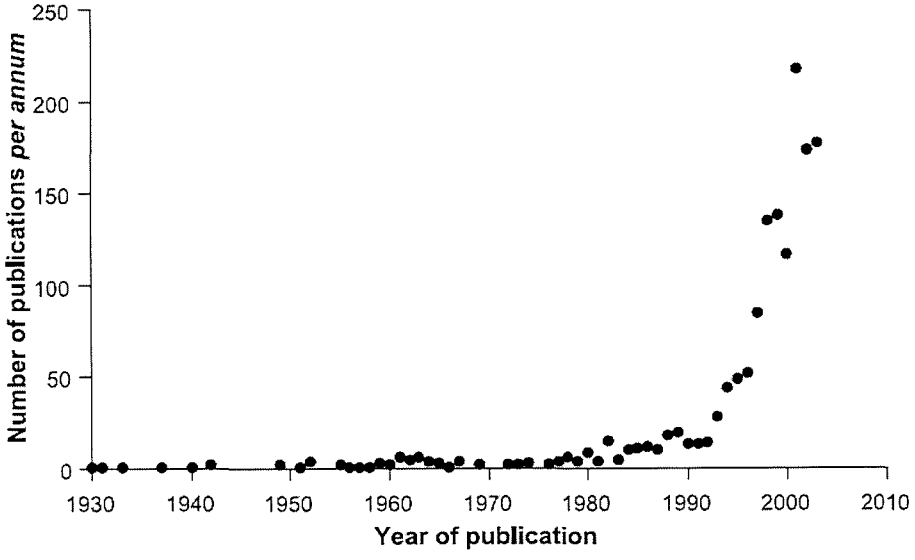


Figure 5-2 Number of MIP publications from 1930 to 2003.

5.3 Synthesis of MIPs

There are a few methods for the preparation of MIPs which are based on the recognition site functionality, such as covalent, non-covalent, semi-covalent imprinting *etc.* Covalent imprinting employs reversible covalent bonds between templates and polymerizable groups during polymerization (Figure 5-3). After polymerization, the templates are removed by cleaving the bonds and the functionality in the binding site is capable of rebinding with target molecules by the same covalent interaction.

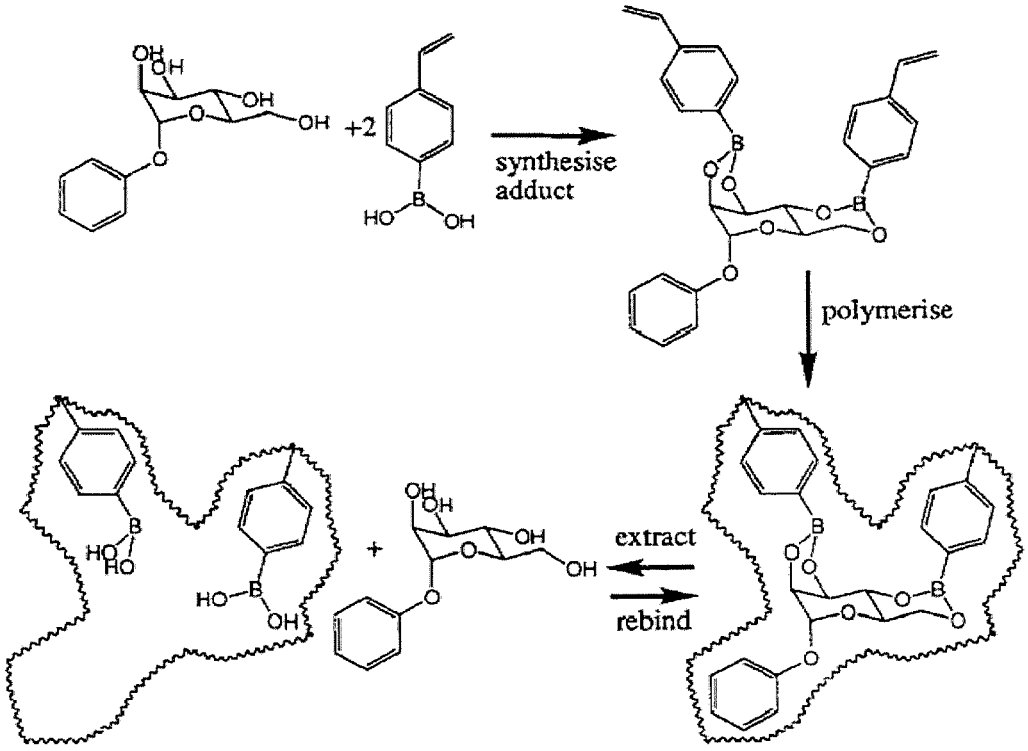


Figure 5-3 Schematic representation of the molecular imprinting *via* the covalent approach [69].

In non-covalent imprinting (Figure 5-4), the interactions between templates and polymers are developed based on non-covalent forces such as H-bonding, ion-pairing and π-π stacking. Templates are removed by disrupting the interactions. Practically, the use of a non-covalent method is simpler and more versatile; therefore, it was adapted in this study.

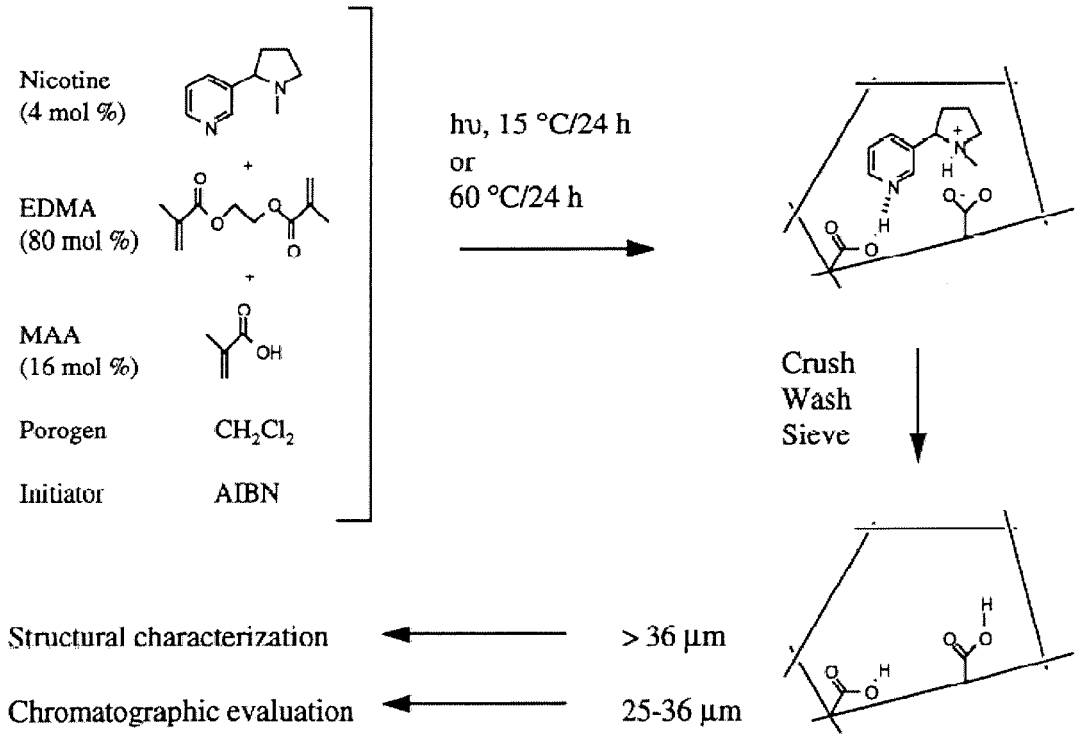


Figure 5-4 Schematic representation of the molecular imprinting *via* a non-covalent approach [88].

5.3.1 Non-covalent imprinting

Most of the non-covalent molecular imprinting methods involve synthesis by free radical polymerisation. It is important to select a suitable template, functional monomer, cross-linker, porogen and polymerization conditions in order to obtain an MIP that will not only be able to rebind with the analyte of interest but also be applicable for a particular sample type.

5.3.1.1 Templates

Templates play an important role in creating rebinding cavities. In order to gain a successful imprinted polymer, the templates must be compatible with other components and must not bear any functional group that has a potential to inhibit the polymerization process. The templates must also be stable at elevated temperature or to the exposure to UV radiation that is normally applied to initiate the polymerization.

5.3.1.2 Functional monomers and Cross-linkers

Functional monomers create the binding interactions in the imprinted sites. In non-covalent imprinting protocols, the template and monomer ratios of 1:4 are normally used [89]. Cross-linkers are necessary to control the morphology of the polymer matrix, to stabilize the imprinted binding sites and to give a high mechanical stability to the polymer. A high degree of crosslinking (70-90%) is essential for achieving specificity. Currently, the majority of the MIPs prepared via non-covalent approaches was synthesised by using vinyl or acrylic groups as imprinting matrix. Typical functional monomers such as carboxylic acids (acrylic acid, methacrylic acid, vinylbenzoic acid), sulphonic acids (acrylamido-methylpropanesulphonic acid), and heteroaromatic (weak) bases (vinylpyridine, vinylimidazole) and commonly utilised cross-linkers such as ethylene glycol dimethacrylate (EGDMA) and trimethylolpropane trimethacrylate (TRIM) are shown in Figure 5-5 and Figure 5-6.

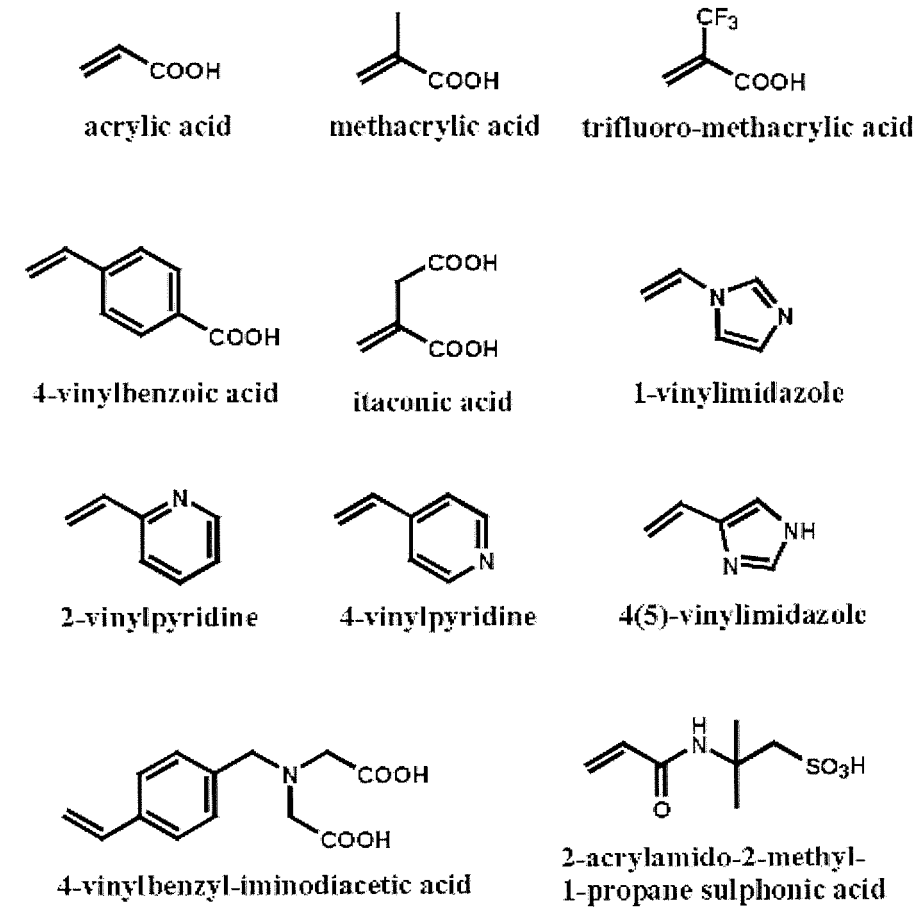


Figure 5-5 Selection of monomers used in non-covalent approach.

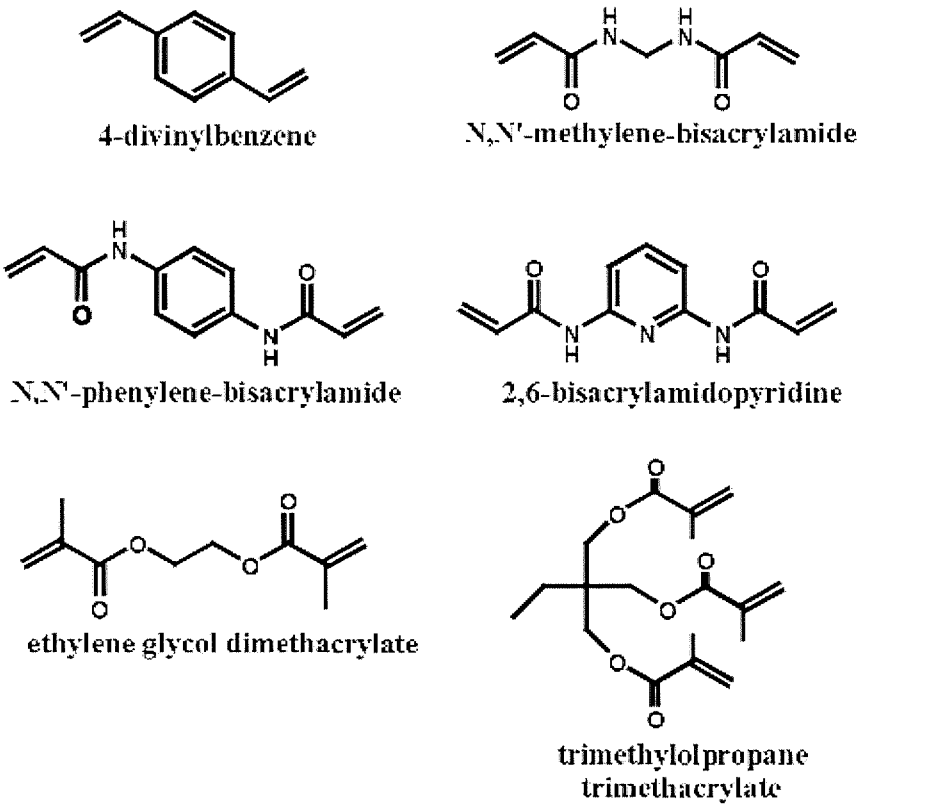


Figure 5-6 Selection of crosslinkers used in non-covalent approach.

5.3.1.3 Porogens

The solvent plays an important role in the outcome of a molecular imprinting process. The solvent must be able to solubilise the constituents and facilitate the complex formation between template and monomer (s). Furthermore, in addition to its influence on porosity and surface area of the resultant polymer, the solvent should govern the strength of non-covalent interactions. The ideal imprinting porogens are apolar solvents which possess low dielectric constant. Polar porogens will inevitably weaken the interaction forces formed between the templates and the functional monomers resulting in poorer recognition.

The specific surface area and the mean pore diameter of the prepared polymers are also dependent on the type of porogen used. A fairly polar solvent such as acetonitrile (dielectric constant, $\varepsilon = 36$) leads to more macroporous polymers than chloroform ($\varepsilon = 5$). A larger surface area and more macroporosity will facilitate mass-transfer of analytes to and from the binding sites, hence lead to a better recognition.

During polymerisation, the solvating properties of the solvent are believed to adjust the shape of the binding sites and the distances between the functional groups in the binding sites. The volume of porogen used in polymerisation is normally half of the total volume of the mixture.

5.3.1.4 Initiators

Free radical initiators such as azobisisobutyronitrile (AIBN) are generally preferred in molecular imprinting due to their electrical neutrality, thus they are less selective. Therefore, the imprinting process can be carried out with various solvents, pH conditions and polarities. These possibilities increase the chances of developing optimised interactions between monomer and template.

Initiation of free radical polymerisation can take place *via* photolysis (UV) or thermolysis. The conversion of methyl methacrylate monomer by AIBN in the initiation step in radical polymerisation is illustrated below in Figure 5-7.

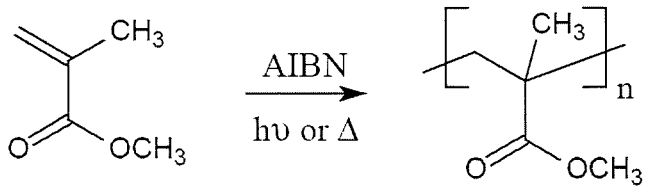


Figure 5-7 Conversion of methyl methacrylate monomer into poly(methyl methacrylate) using AIBN in free radical polymerization.

5.4 Applications of MIPs

The preparation of MIPs is rather easy and relatively cheap. Apart from its ability to recognize the target analyte in a similar way to biomolecule-based recognition, an MIP also exhibits high physical and chemical resistance toward various factors such as pH, mechanical stress, elevated temperature and pressure, acid and bases, organic solvents and metal ions. It can be stored at ambient temperature for several years without loss of performance. These advantages have led to the development of various uses, for example, as antibody and receptor binding mimics for assays and sensors and as separation materials.

There are several reports on the application of imprinted polymers as substitutes for natural antibodies in immunoassays. Mosbach's group reported the development of an MIP-based immunoassay against theophylline and diazepam [90]. Andersson *et al.* proved that MIP-assays could also be performed directly with diluted blood plasma [91]. Binding characteristics in terms of affinity and specificity were similar to those of antibodies and natural receptors.

Haupt *et al.* imprinted a polymer with a 2,4-D herbicide and used it as an artificial recognition element [92]. The method demonstrated that a radioligand binding assay using imprinted polymer particles is as sensitive as an indirect ELISA or RIA assays.

The difficulties in operating biosensors due to a lack of stability in the natural biolomolecules also led to the development of imprinted polymers as a replacement. Kriz and Mosbach constructed a morphine-sensitive device by entrapping morphine imprinted polymer particles in an agarose layer around a platinum electrode [93]. Although the response time of the sensor was slow, this study demonstrated the potential of an imprinted polymer as a sensing material as it was shown to have long-term stability and to be highly resistant to harsh chemical environments.

The group of Piletska developed synthetic receptors for cocaine, deoxyephedrine, methadone and morphine [94]. The imprinted polymers were designed using computational modelling in order to choose the best monomers, indicate the template-monomer reaction and optimise the composition of the polymers. All the MIPs thus prepared demonstrated good selectivity compared to blank polymers. The recognition of the synthetic receptors was found to be similar to that of the natural molecules, leading the author to recommend these artificial receptors for investigation in sensor platforms.

The applications of MIP in separation techniques have been by far the most extensively studied ones, especially as stationary phases for LC and as SPE sorbents. In both applications, the imprinted monolithic polymer is usually crushed, extracted and sieved to appropriate particle sizes for subsequent packing in either chromatography columns or SPE cartridges. The applications of imprinted polymers in chromatographic systems mainly focused on their ability to distinguish between the enantiomers of a drug of interest. Hart *et al.* reported the separation of chiral benzodiazepines in HPLC analysis [95]. The MIP stationary phase in their study showed significant dependence on the chiral match or mismatch with the shape of the imprinted binding site. Another example

of the application of an MIP as a selective affinity medium has been reported by Matsui and Takeuchi [96]. This study examined the separation of nicotine in an LC system by using anti-nicotine prepared with TFMAA and MAA as a functional monomer. The imprint polymer rod prepared with TFMAA was found to display a high affinity chromatographic selectivity.

The development of Molecularly Imprinted Solid Phase Extraction (MISPE) significantly indicates the potential of MIP applications in affinity separation studies, particularly due to their high separation factors and resolution performances [97, 98, 99]. The development, application and limitations of MISPE will be further discussed in the following section.

5.5 MISPE

The study of MISPE was first described by Sellergen in 1994 for the extraction of pentamidine from urine samples [100]. A clean extract and enrichment of the sample was achieved, thus enabling the analysis of the sample by direct injection. Martin *et al.* developed a propanolol-derived MIP and evaluated its extraction ability for aqueous samples [101]. They highlighted the necessity of applying suitable ion-modifiers in the elution step in order to obtain good recovery.

The first MISPE developed for herbicide analysis was reported by Muldoon *et al.* in 1997 [102]. An anti-atrazine MISPE polymer was used to extract the herbicide from beef liver samples. The use of an apolar solvent in the washing step gave a better recovery and low non-specific binding to the polymer. Another anti-atrazine MISPE was developed by Matsui *et al.* in order to extract the herbicide in environmental analysis [103]. They prepared the polymer by using suspension polymerization in contrast with bulk polymerization reported in previous work, obtaining uniformly-sized and bead-shaped particles. The optimised protocol enabled better sensitivity and high recovery of the herbicide.

5.5.1 MISPE Protocol

MISPE is based on conventional SPE procedures which require conditioning, loading, clean-up and elution steps. In the conditioning step, the binding sites of the MIP are usually activated with solvent to maximise the interactions with the target analyte.

The clean-up of analytes by MISPE can be achieved by either a reversed-phase or normal-phase extraction protocol. In the reversed-phase protocol, the usually non-selective adsorption step occurs in the loading step; hence selective extraction takes place in the washing procedure in which the correct solvent must be chosen to remove non-selectively bound matrix components. The analyte is recovered by eluting with a solvent of high eluotropic strength.

In the normal-phase protocol, adsorption takes place in the loading step. Samples in an aqueous matrix are normally transferred to organic solvent via LLE or SPE with organic eluting solvent prior to loading. When loaded, the extracted organic materials will be selectively retained and some of the matrix compounds will be eluted. The remainder of the matrix compounds will be further removed in the washing step prior to the elution step.

The choice of solvent is important in the recognition step. As a rule of thumb, the most appropriate recognition solvent should be more or less identical to the imprinting porogen in order to obtain the best recognition. The use of the imprinting porogen in the recognition step will also enable retention of the morphology of the polymers and recreate the dimensions of the binding sites formed during the polymerisation. The morphology is dependent on the surrounding medium. In chlorinated solvents, such as chloroform and dichloromethane, the polymers will swell and this will lead to changes in the three-dimensional configuration of the functional groups taking part in the recognition sites, resulting in poorer binding capability.

The analyte can be efficiently desorbed from the phase by using a highly polar solvent. In methods dealing with strongly bound analytes, the addition of a small amount of acid such as acetic acid or trifluoroacetic acid, or base such as triethylamine in the polar solvent is sufficient to suppress the binding.

Similar to conventional SPE, MISPE can be performed in off-line and on-line modes in which the system is normally coupled to a chromatographic technique [104, 105, 106, 107]. In the off-line mode, the imprinted polymer is packed in polyethylene cartridges while in the on-line mode the polymer is packed in an LC pre-column. By far, there have been more studies reported using the off-line mode, mainly due to its simple procedure and instrumentation. This mode also allowed the use of stronger elution conditions which sometimes is not compatible with the analytical instrumentation.

Furthermore, in cases where the porogenic or organic solvent is used as a wash solvent, the MIP needs to be dried in order to overcome the immiscibility to aqueous system during the loading step. This drying step is difficult to combine with the on-line procedure.

5.5.2 Advantages and Limitations of MISPE

The use of MISPE is advantageous in performing selective extraction where it is able to preconcentrate and separate target analytes from the matrix compounds. Theoretically, this will lead to a cleaner extract, diminished interferences during analysis and subsequently leading to increased sensitivity. The MISPE protocols are also simpler and easier compared to conventional SPE.

The main limitation of MISPE is the bleeding of the template which may lead to false positives in the quantitation of the template in real samples. However, this problem could be overcome by using an analogue molecule which is structurally related to the target analyte. Andersson *et al.* reported the synthesis of an MIP using a template that was a close structural analogue of the target analyte, sameridine [108]. The MIP was able to extract sameridine selectively and curtailed the problem caused by template bleeding. For cases where there is no suitable analogue molecule, a few choices for template removal were reported, including thermal annihilation, microwave-assisted extraction and desorption with supercritical fluids [109].

Another problem in dealing with MIPs is due to its physical form. Normally, the preparation of the MIPs *via* a bulk polymerization process produced a rigid monolith that requires grinding and sieving prior to use. Unfortunately, the particles obtained are of irregular size and shape. Therefore, several techniques have been investigated to prepare more homogeneous MIP particles [110, 111, 112].

5.6 Aims

The following research work was intended to produce an anti-diazepam MIP by adapting the synthetic method that was previously reported by Vlatakis *et al.* and which produced an MIP which demonstrated good selectivity in immunoassay [90]. In the following study, an MIP was prepared and applied as an SPE sorbent in a MISPE protocol for diazepam and its analogues from hair samples. By using the same synthesis

method, an anti-flunitrazepam MIP was also produced, applied as an SPE sorbent and its performance was compared to the anti-diazepam MISPE.

The final aim was to synthesis a paraquat MIP using an analogue molecule as a template. The MISPE for paraquat and other QA herbicides was then established.

6 MOLECULARLY IMPRINTED SOLID-PHASE EXTRACTION (MISPE) OF BENZODIAZEPINES FROM HAIR SAMPLES

6.1 Benzodiazepines

Benzodiazepines are the most commonly prescribed drugs for the treatment of anxiety and insomnia in the general population [113]. They are also used as sedatives and muscle relaxants before surgical and medical procedures. Chlordiazepoxide (Librium®) is the earliest benzodiazepine available in the market followed by diazepam (Valium®) production in 1963 and Valium became the most commonly prescribed benzodiazepine in Scotland [114]. Currently, newer and shorter-acting benzodiazepines such as lorazepam (Ativan®), oxazepam (Serax®) and temazepam (Normison®, Restoril®) are commonly prescribed. Other benzodiazepines which are also commonly prescribed as hypnotic agents in many European countries are nitrazepam (Mogadon®) and flunitrazepam (Rohypnol®).

6.1.1 Mode of action and adverse effects of benzodiazepines

Benzodiazepines are agonists for gamma-aminobutyric acid (GABA), which is an inhibitory neurotransmitter. The interaction of benzodiazepines with GABA receptors will increase chloride ion movement through ion channels into the cytoplasm of neurones, causing hyperpolarisation and a reduction in the activity of certain key areas in the central nervous system (CNS) [95, 115]. Enhanced GABA activity will create an anxiolytic, hypnotic and anticonvulsant effect.

Benzodiazepines can very occasionally cause paradoxical excitement. The user become more anxious, aggressive and easily irritated. They will also experience insomnia, nightmares and hallucinations. Long-term users are often depressed and experience emotional anaesthesia, where they are unable to feel any pleasure or pain.

Polydrug users, drug abusers who administer more than one drug at the same time, also called polypharmacy, will face a greater risk of fatality when taking benzodiazepines with other drugs including antidepressants, tranquilisers, opiates and alcohol. Benzodiazepines are also known to cause memory impairment and oversedation, which

may contribute to accidents when handling machinery at work place and traffic accidents.

6.1.2 Pharmacokinetics of benzodiazepines

This study focuses on nine benzodiazepines, namely diazepam, nordiazepam, oxazepam, temazepam, lorazepam, chlordiazepoxide, nitrazepam, flunitrazepam and 7-aminoflunitrazepam. The molecular structures of these drugs are shown in Figure 6-1, while the pharmacokinetic properties of the drugs are summarised in Table 6-1.

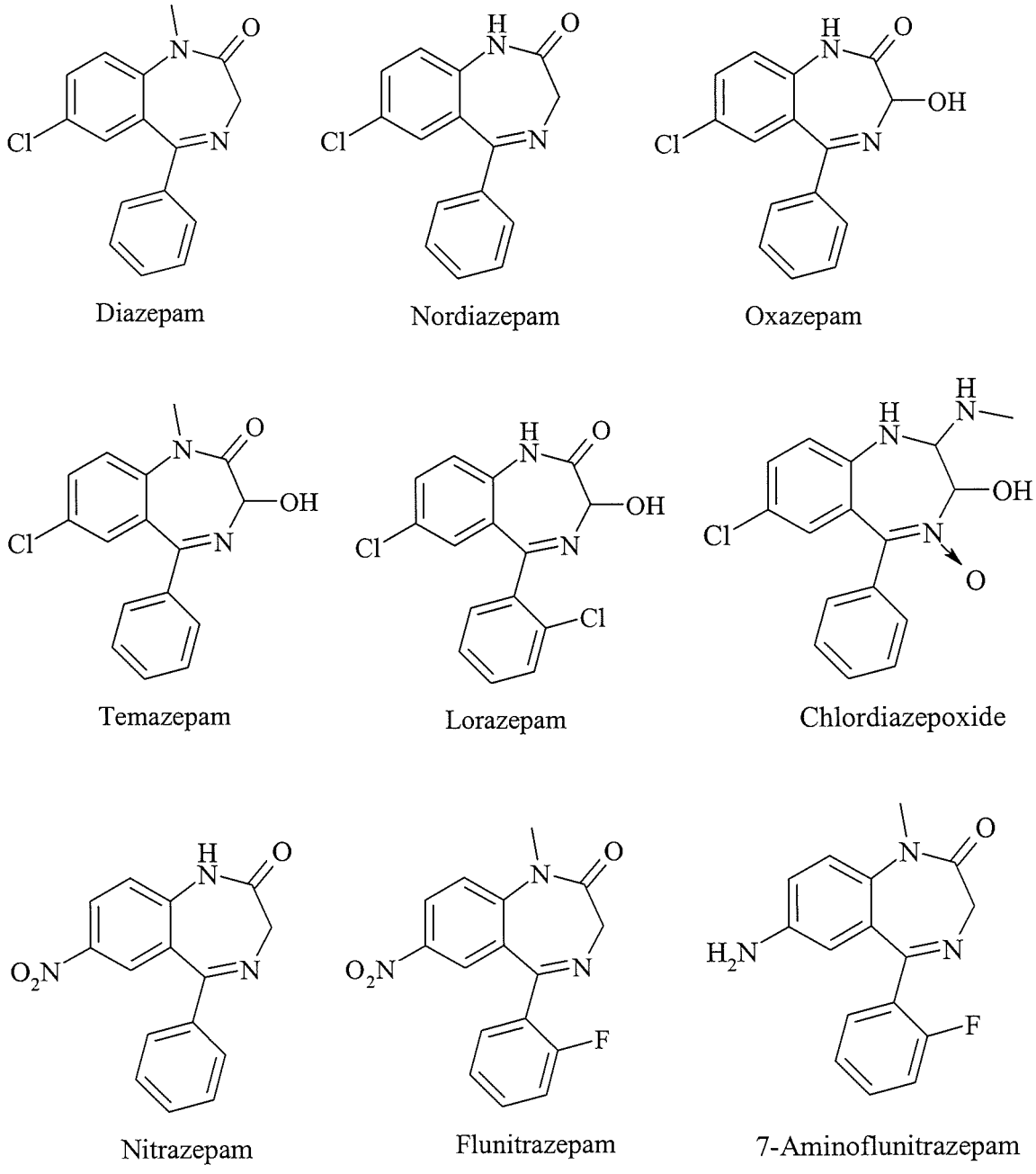


Figure 6-1. Chemical structures of benzodiazepine derivative drugs.

Table 6-1 Pharmacokinetic properties of benzodiazepines

	Half Life (hr)	Vd (L/kg)	Protein Binding (%)	pKa
Chlordiazepoxide	6-27	0.3-0.5	94	4.8
Diazepam	21-37	0.7-2.6	96	3.4
Nordiazepam	31-97	-	97	3.5, 12.0
Oxazepam	4-11	0.7-1.6	87-94	1.7-11.6
Temazepam	3-13	0.8-1.0	97	1.3
Lorazepam	9-16	0.9-1.3	80	1.3, 11.5
Nitrazepam	17-48	2-5	87	3.2, 10.8
Flunitrazepam	9-25	3.4-5.5	78	1.8

6.1.3 Metabolism and Excretion

When consumed, chlordiazepoxide produced 2 major metabolites which are norchlordiazepoxide and demoxazepam [16]. The reduction of demoxazepam will form nordiazepam which is normally accumulated in the plasma of chronic users. Another active metabolite, oxazepam, is formed following the hydroxylation of nordiazepam.

Diazepam is metabolized in the liver to produce nordiazepam *via* N-demethylation [16]. Both of these compounds are converted further to oxazepam and temazepam *in vivo*. In urine, traces of diazepam and nordiazepam are found. About 33% of the dose is excreted in urine as oxazepam glucuronide and another 20% as conjugates of nordiazepam, 4'-hydroxydiazepam and temazepam.

Lorazepam is conjugated with glucuronic acid to form an inactive metabolite, lorazepam glucuronide [16]. Temazepam will be converted to oxazepam. Both nitrazepam and flunitrazepam are metabolized via reduction of the nitro group to produce amino metabolites, 7-aminonitrazepam and 7-aminoflunitrazepam, respectively [16]. Following a single administration of a 2 mg oral dose of flunitrazepam, the concentration of 7-aminoflunitrazepam reaches 50-200 μg/mL in urine within 6-24 hours [16].

6.1.4 Benzodiazepines Abuse

Benzodiazepines are very addictive, cheap and widely available on the street [116]. Therefore these drugs are taken for recreational purposes by drug abusers together with other illicit drug(s) or alcohol [117]. Polydrug abusers take benzodiazepines to enhance the 'high' induced by the primary drug of abuse such as opioids, cocaine and amphetamines. They are also taken when there is a shortage of the user's primary drug to alleviate withdrawal effects or alternatively, benzodiazepines are taken to calm the user down from 'upper' effects caused by stimulants. One scenario is the use of diazepam to help a user sleep after a weekend binge on ecstasy [118]. Chronic alcohol users use a mixture of benzodiazepines and alcohol to promote a hedonic effect [119].

Flunitrazepam is reputedly involved in drug-facilitated sexual assault (DFSA) and other drug-facilitated crime cases [120, 121]. It is formulated as a colourless, odourless and tasteless tablet. The drug is also used by the assailant due to its ready solubility in the victim's drink and rapid onset of action, *i.e.* approximately 15-20 minutes after administration, and the effect lasts about 4 to 6 hours [121]. The victim also has a problem in recalling the assault because of the drug amnesic effect (retrograde amnesia).

One study into drug related deaths in the Strathclyde region of Scotland between 1995 and 1998 found that 45% of cases tested positive for diazepam while 33 % was positive for temazepam [122]. Temazepam was formerly the benzodiazepine of choice of heroin users, who co-administered the two drugs. Due to this abuse, temazepam was largely withdrawn in the area as a prescribed drug [122]. An assessment of drug prevalence in impaired and fatally injured drivers in the same region during the same period found benzodiazepines to be present in 82% of the cases [123]. Another study showed that 0.8% of the population in Scotland aged between 15 and 54 were identified as opiate or benzodiazepine users [124].

Benzodiazepines are detected very frequently in the five Nordic countries i.e. Denmark, Finland, Iceland, Norway and Sweden in year 2002, with diazepam and flunitrazepam being the commonest benzodiazepines detected in drug-related deaths [125]. In Singapore, diazepam, nitrazepam and midazolam were reported to be the most frequently abused drugs, used concurrently with burprenorphine in fatal cases [126].

6.2 Hair Samples

In general forensic drug analysis, sampling of tissues and body fluids such as blood and urine are very common. These samples are however, only able to provide information of drug exposure within 1-5 days prior to sample collection [127]. Therefore, the use of other unconventional samples such as hair and nail become a more important practice to gain long-term information on the magnitude of drug use. Sectional analysis of hair samples will also provide a reliable guide of the duration of drug ingestion.

The collection of hair samples is also less invasive than for blood or urine. Hair is easy to collect, store and dispatch compared to blood and urine samples. Hair samples become more important when dealing with autopsy and drug-facilitated crime cases, especially when the crimes are reported more than 24 hours after consumption, where blood and urine samples are no longer appropriate.

6.2.1 Incorporation of Drugs in Hair

A single strand of hair consists of a root and a shaft. The root contains a hair bulb and follicle, while the shaft consists of medulla, cortex and cuticle (Figure 6-2).

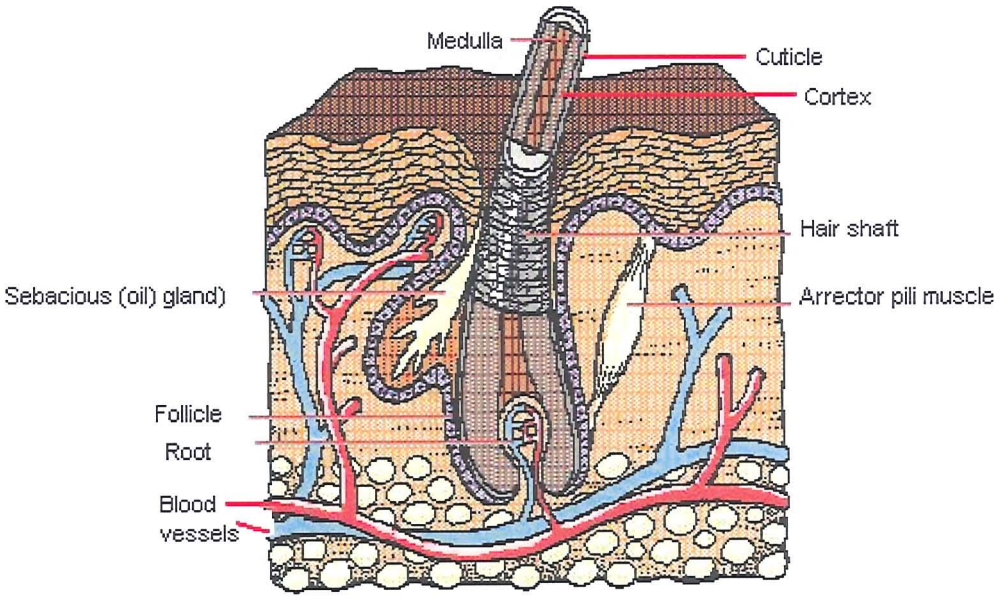


Figure 6-2 Hair structure [128].

When ingested, drugs will circulate in the bloodstream including the blood vessels of the hair bulb, where it can diffuse out and become entrapped in the core of the hair shaft as it grows out from the hair follicle. Normal growth rates for human hair are approximately 1 to 1.5 cm per month although the rate is dependent on other factors including anatomical location, and the race, sex and age of the person [127, 129]. The life span of a single hair varies from about 4 months to 4 years.

6.2.2 Analysis of Benzodiazepines in Hair

Benzodiazepine detection has been reported in hair collected from living and deceased users by HPLC [130, 131], GC-MS [132, 133, 134, 135] and LC-MS-MS [136, 137, 138, 139, 140].

Couper *et al.* established an HPLC method for the detection of psychotropic drugs including benzodiazepines in hair [130]. However, due to the lack of sensitivity of the method, diazepam, nitrazepam and oxazepam were not detected in hair samples obtained from volunteers under treatment. Staub and El Mahjoub reported the use of an on-line restricted access extraction column coupled with an HPLC-Diode Array Detector system [131]. The limit of detection (LOD) was determined to be about 0.2 ng/mg hair for all selected benzodiazepines with acceptable recovery values. This method provided a rapid analysis which made it suitable for routine analyses. The authors also suggested that detection using mass spectrometry method should be able to provide better results.

In the middle to late 1990s, most of the analyses of benzodiazepines in hair samples were carried out using GC-MS, which is a more sensitive and specific method. The detection of nordiazepam and oxazepam was first reported by Kintz *et al.* in 1996 using gas chromatography with negative-ion chemical ionisation (GC-NCI) mass spectrometry [132]. The LOD values of nordiazepam and oxazepam were 0.01 and 0.0005 ng/mg respectively. The percentage recovery values at 2 ng/mg hair were 77.3 and 68.6 %, respectively. GC coupled with NCI proved to be a more sensitive method compared to EI or positive-ion chemical ionisation (PCI).

There were more studies subsequently reported on the use of GC-NCI for benzodiazepine analysis in hair samples [133, 134, 135]. All of these studies provided very sensitive methods with LOD values ranging from 1-20 pg/mg hair. These methods were found to be suitable for screening and confirmation purposes.

Yegles *et al.* developed a method for the detection of benzodiazepines and other psychotropic drugs in human hair by GC-MS [141]. The LOD values for the benzodiazepines ranged between 0.01-2 ng/mg hair, suggesting that this method is suitable for screening purposes.

Recently, after the commercial development of successful interfaces for the coupling of liquid chromatography with MS, reports on the use of GC-MS for benzodiazepine detection have gradually decreased. The LC-MS technique was then favoured due to its soft ionisation and mild separation conditions compared to GC-MS, in which some compounds were thermally decomposed to produce products which were similar to metabolites of the benzodiazepines [142]. Furthermore, LC-MS requires a simpler sample preparation procedure without the need for a derivatisation step in order to get a compound that is compatible with GC.

The most common extraction methods used for hair samples prior to LC-MS analysis involve LLE and SPE. Chèze *et al.* used dichloromethane to extract benzodiazepines from hair [136]. The LOD values for bromazepam, clonazepam and 7-aminoclonazepam were 1-2, 0.5 and 2 pg/mg hair respectively. Irving *et al.* also reported the use of dichloromethane as a solvent in LLE followed with LC-MS-MS analysis [137]. Kintz *et al.* and Villain *et al.* used a mixture of dichloromethane and ether as an organic solvent for LLE [138, 139]. The LODs for most tested benzodiazepines were equal to or less than 2 pg/mg hair. For bromazepam, clonazepam and 7-aminoclonazepam, the LOD values were similar to the work reported earlier [136].

Kronstrand *et al.* developed an extraction method using BondElut Certify™ SPE columns followed with LC-MS-MS analysis [116]. The method has been applied to human hair samples obtained from forensic and clinical psychiatric patients. The percent recovery values of between 75 and 99% were obtained for all tested benzodiazepines.

Miller *et al.* also reported the use of SPE methods for hair extraction [143]. Percentage recoveries ranged between 55-94% with good LOD values from 0.03 to 0.62 ng/30 mg hair.

6.3 Aim of this Study

The aim of this study was to establish a simple, rapid and cleaner analysis of benzodiazepines in hair sample using MISPE.

6.4 Experimental Section

6.4.1 Instrumentation

LC-MS-MS analyses of benzodiazepines were carried out using a Thermo Finnigan LCQ Deca XP (Thermo Finnigan, San José, CA, USA) equipped with a Surveyor autosampler and MS pump system. Chromatographic separation was performed using a Gemini C18 column (150 mm x 2.0 mm ID, 5 μm particle size) and a 4.0 mm x 2.0 mm, 5 μm guard column (Phenomenex, Torrance, CA, USA) with the same packing as the column.

6.4.2 Chemicals and Materials

Diazepam for polymer synthesis was obtained from Roche (Hertfordshire, UK). Methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) were from Aldrich (Steinhem, Germany), chloroform was from Rathburn (Peebleshire, UK) and 2,2'-azobisisobutyronitrile (AIBN) was from Acros Organics (Geel, Belgium).

Ammonium formate and sodium dodecyl sulphate were from Sigma-Aldrich (Dorset, UK). HPLC grade acetonitrile, formic acid, toluene, chloroform and dichloromethane were purchased from BDH (Poole, UK).

Empty 1mL polyethylene SPE cartridges and frits (20 μm mean pore size) were purchased from International Sorbent Technology Limited (Mid Glamorgan, UK).

6.4.3 Standards

For LC-MS-MS confirmation, diazepam, nordiazepam, nitrazepam, chlordiazepoxide, temazepam, oxazepam, lorazepam, flunitrazepam and 7-aminoflunitrazepam and the internal standards diazepam-d5, nordiazepam-d5, temazepam-d5, oxazepam-d5, lorazepam-d4, flunitrazepam-d7 and 7-aminoflunitrazepam-d7 were purchased from Promochem (Teddington, UK). It was not financially viable to use the chlordiazepoxide-d5 internal standard therefore oxazepam-d5 was selected for

chlordiazepoxide quantitation since its retention time (t_R) was close to the chlordiazepoxide t_R (5.1 and 6.2 min respectively). The m/z of the nitrazepam-d5 internal standard precursor ion is the same as the m/z of the oxazepam precursor ion (m/z = 287) and both have a similar t_R of 5.8 min, resulting in undesirable co-elution. Therefore oxazepam-d5 was used as the internal standard in nitrazepam quantitation.

6.4.4 Preparation of Mobile Phase

A mobile phase consisting of 3 mM ammonium formate and 0.001% formic acid in water was prepared by adding 0.189 g ammonium formate and 10 μl of concentrated formic acid to a 500 mL volumetric flask and making up to 500 mL with deionised water.

6.4.5 Preparation of Solvents for the Synthesis of the Imprinted Polymers

6.4.5.1 Porogen - Ethanol-free chloroform

Ethanol-free chloroform was prepared by washing HPLC grade chloroform twice with an equal volume of water in a separating funnel. The chloroform layer was collected and dried over anhydrous calcium chloride. The chloroform was then filtered and collected in a round bottom flask. Further drying was then carried out over phosphorus pentoxide by refluxing the chloroform in a Soxhlet extractor at 70°C. Ethanol-free chloroform was collected and put in an amber glass bottle with activated 4 Å molecular sieve.

6.4.5.2 Cross-linker - EGDMA

EGDMA was washed with 10% aqueous NaOH in brine. It was then dried over anhydrous sodium sulphate and distilled under reduced pressure.

6.4.5.3 Monomer - MAA

MAA was dried over anhydrous sodium sulphate and distilled under reduced pressure.

6.4.5.4 Initiator - AIBN

AIBN was dissolved in methanol at room temperature, then the solution was filtered and kept in a freezer to recrystalize.

6.4.6 Synthesis of Diazepam MIP

The MIP for diazepam was prepared using the method described by Vlatakis *et al.* by dissolving diazepam (0.2202 g, 0.77 mmol), MAA (0.3996 g, 4.64 mmol) and EGDMA (4.6004 g, 23.21 mmol) in ethanol-free chloroform (6.7 mL) in a 25 mL, thick-walled glass Kimax tube [90]. AIBN (0.0838 g, 0.51 mmol) was then added and the solution sparged with oxygen-free nitrogen for 5 min while cooling in an ice bath. The sealed Kimax tube was thermostated at 4°C, to facilitate template-monomer complex formation, and irradiated with a Blak-Ray longwave UV lamp (model B-100A) for 24 hours. Thereafter, the polymer monolith obtained was transferred to a water bath set at 60°C for a further 24 hours, to complete the curing of the polymer. A non-imprinted polymer (NIP) was prepared in the same manner as the MIP but in the absence of template (diazepam). The interaction between the template and monomer and the schematic diagram of the polymerization procedure are illustrated in Figure 6-3 and Figure 6-4.

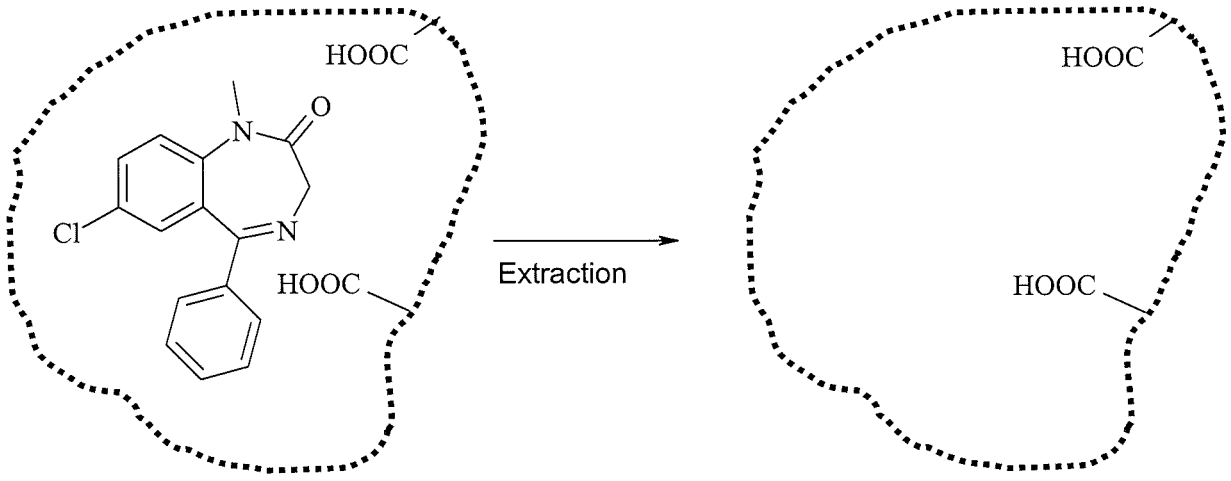


Figure 6-3 Illustration of template-imprinted polymer and its recognition.

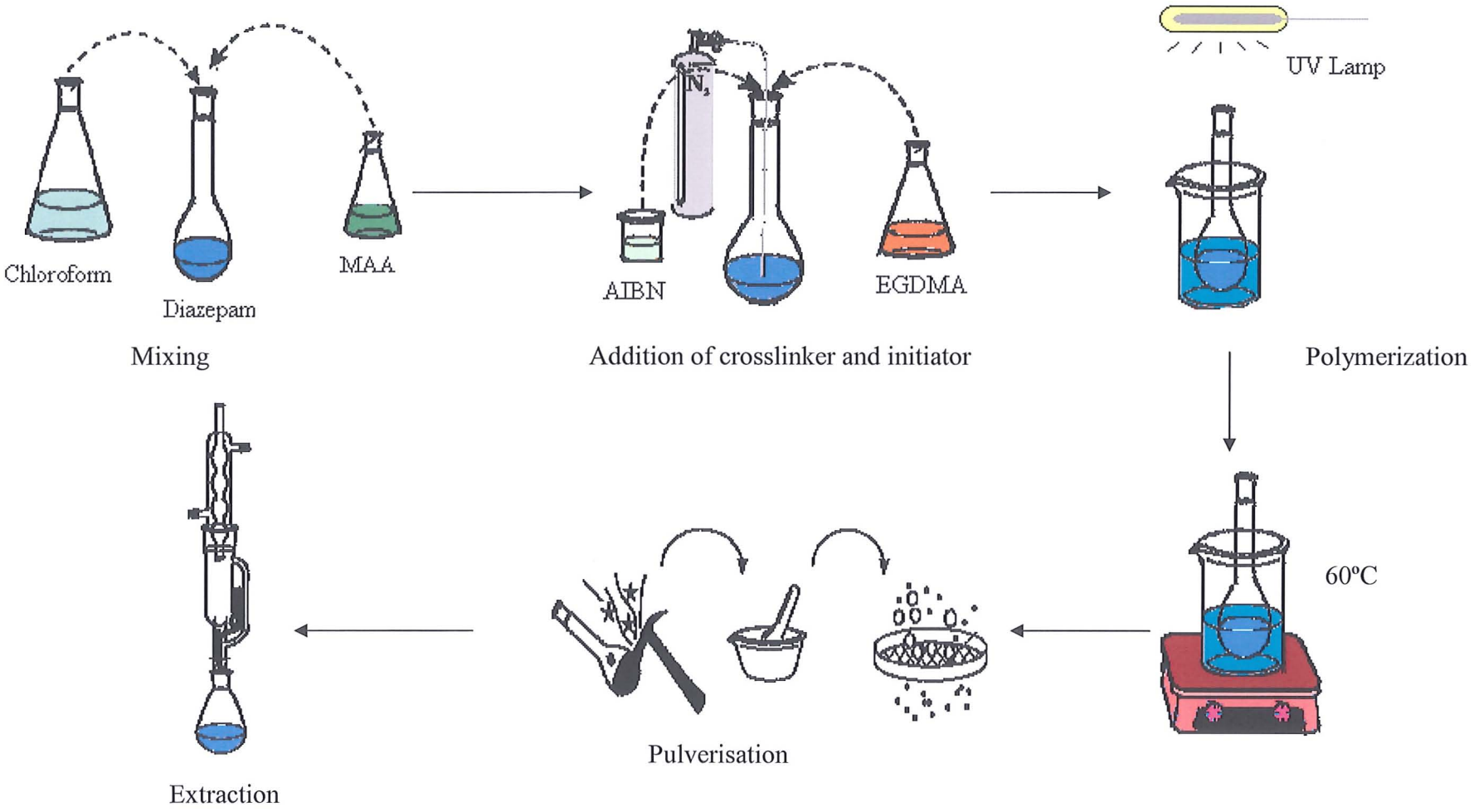


Figure 6-4 Schematic illustration of the polymerization procedure.

The MIP and NIP monolith were crushed, mechanically ground and wet-sieved using acetone to deliver polymer particulates in the 25-38 μm size range. The template was extracted from the MIP by extensive washing with a mixture of methanol and acetic acid (9/1, v/v) for 24 hours. The polymer particles were then dried under vacuum at 60°C prior to use. The resulting polymer particles prepared using a bulk polymerization process are irregular in size and shape as depicted in Figure 6-5 below.

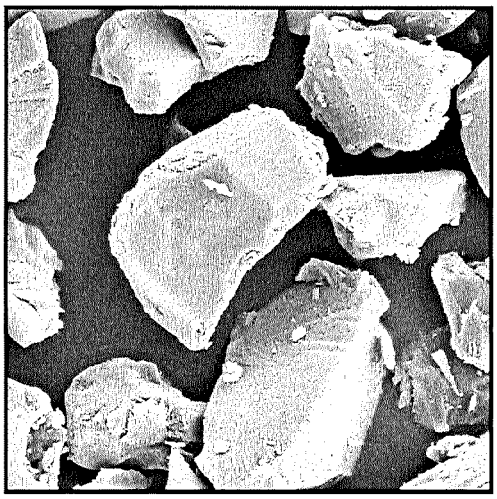


Figure 6-5 The diazepam MIP obtained using by bulk polymerization process scanned with SEM (x 1000).

6.4.7 Preparation of MISPE Cartridges

20 μm polyethylene frits were inserted into empty 1 mL polypropylene SPE cartridges. The cartridges were connected to a vacuum manifold and 20 mg of the MIP or NIP were slurried with acetonitrile and packed into the cartridges. The cartridges were subjected to vacuum for 30 s before insertion of a second frit on top of the sorbent bed.

As observed in previously published work, there was still small amount of template remaining in the MIP even after extensive washing with acid and organic solvent via Soxhlet extraction,. In this case, about 8 ng diazepam was retained in 20 mg polymer. In order to remove the template totally, the MIP cartridge was treated several times in series with 1 mL acetonitrile, 1 mL chloroform and 1 mL of 30% acetic acid in acetonitrile. After each treatment cycle, the 30% acetic acid/acetonitrile fraction was collected, evaporated and reconstituted with LC-MS mobile phase prior to analysis. In between treatment series, the MIP sorbent was allowed to dry.

The results show that the template was completely removed from the imprinted polymer when treated with washing solutions more than eight times (Figure 6-6). Therefore, all the cartridges were treated with these solutions prior to being used in the extraction procedure. The hump observed in the graph at the second and seventh treatment can be seen when the polymer is dried overnight. This is probably due to volume changes of the polymer material caused by drying and wetting process. Same observation was also reported when switching between solvents [109].

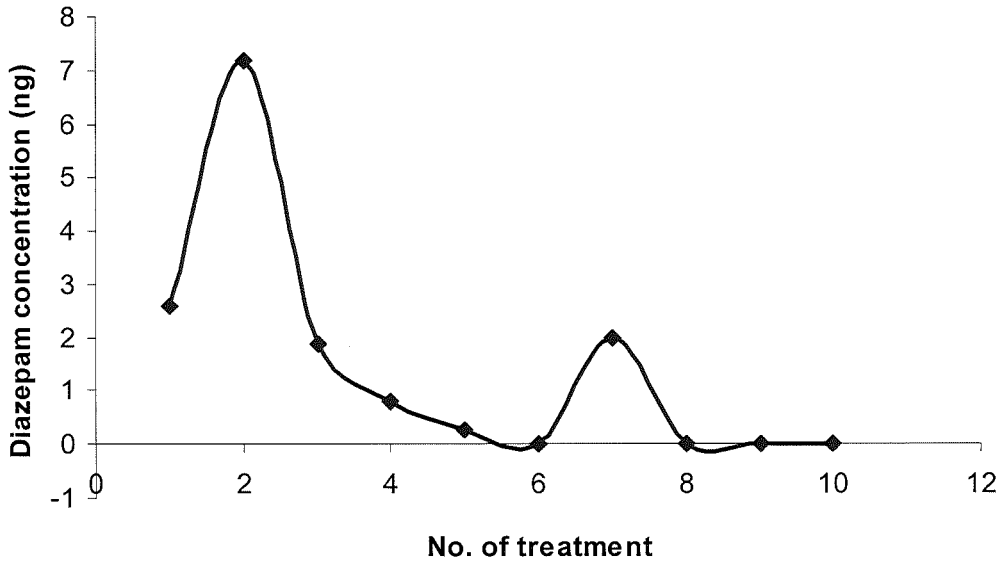


Figure 6-6 Concentration of diazepam in the eluate after each treatment.

6.4.8 LC-MS-MS Analysis

LC was carried out using 3 mM ammonium formate and acetonitrile at a flow rate of 0.3 mL min^{-1}. The elution program consisted of a linear gradient (65-20%) of 3 mM ammonium formate for 13 minutes. The percentage of ammonium formate was decreased to 10% between 13 and 13.5 minutes. It was held at 10% between 13.5 and 16.5 minutes before being increased to the initial condition (65%) between 16.5 and 20 minutes. 20 µl samples were injected into the Gemini C18 column.

All mass spectral data was collected in electrospray positive ion mode. The capillary temperature, sheath and auxiliary gas flow rates and collision energies were optimized for each analyte. The probe voltage used was 4.5 kV. Internal standard data was collected in selected ion monitoring (SIM) mode and analytes were identified on the

basis of their retention time and full MS-MS spectra. The product ion ratios were monitored to gain further qualitative identification data. The optimum tuning parameters, precursor and product quantitation ions are shown in Table 6-2.

6.4.9 Preparation of Hair Samples

Blank hair samples were collected from volunteers. All samples were wrapped in aluminium foil and stored at room temperature. Prior to analysis, hair samples were decontaminated and pre-extracted using the method described by Miller *et al.* [143]. Benzodiazepines are known to be unstable in strong alkaline media, therefore a previously published method was used which involved mild alkaline incubation conditions and which gave good extraction efficiency for diazepam, [144, 145]. The stabilities of the benzodiazepine drugs using these mild alkaline incubation conditions have been described previously [143]. No significant hydrolysis was observed and recovery values ranged between 87-102%, with acceptable precision. Hair samples were washed with 0.1% aqueous sodium dodecyl sulfate (SDS) and sonicated for 10 minutes. The hair samples were then rinsed and sonicated for 10 minutes, twice with deionised water and twice with dichloromethane, then left to dry in air. The dried hair samples were then weighed and approximately 30 mg samples transferred into vials for the hair pre-extraction process.

In the hair pre-extraction process, 1.5 mL of a mixture containing methanol and 25% aqueous ammonium hydroxide (20:1, v/v) was added to each vial containing a hair sample, sonicated for 1 h and left overnight at room temperature. The extraction solvent was removed and transferred from the vial to a test tube. The hair was washed twice more with 0.75 mL of methanol:25% aqueous ammonium hydroxide (20:1, v/v) and the washings removed and transferred to the same test tube as the first extract. The contents of the test tubes were evaporated to dryness under a stream of nitrogen gas and reconstituted with 1 mL of loading solvent prior to extraction with the diazepam MISPE.

Table 6-2 Optimum tuning parameters, precursor and product ions for each analyte

Analyte	Sheath Gas (AU)	Auxiliary Gas (AU)	Capillary Temperature (°C)	Collision Energy (%)	Precursor ion (MH+) m/z	Product ions m/z
7-aminoflunitrazepam	20	20	280	40	284	264*, 256
Chlordiazepoxide	20	20	300	29	300	283*, 241
Diazepam	20	20	300	42	285	257*, 222
Flunitrazepam	20	20	280	43	314	286*, 268
Lorazepam	30	20	290	30	321	303*, 275
Nitrazepam	30	20	300	42	282	254, 236*
Nordiazepam	20	15	300	41	271	243*, 140
Oxazepam	20	20	300	29	287	269*, 241
Temazepam	20	20	300	29	301	283*, 255

***Quantitation ion**

6.4.10 Method Development of MISPE Protocol

The binding properties of the molecularly imprinted sorbent toward the template analyte are influenced by the type of solvent used in the loading, washing and elution steps [146]. In order to achieve good retention and recovery of analytes, the MISPE protocol must be optimised by selecting the appropriate solvent in each extraction step.

6.4.10.1 Selection of elution solvent

In order to choose the best elution solvent the MIP column was first conditioned with 1 mL chloroform (porogen used in MIP preparation) and then loaded with 1 mL of 10 ng diazepam standard solution and washed repeatedly with 1 mL acetonitrile containing 5, 8, 10, 15, 20 and 25% (v/v) of acetic acid, beginning with the eluent containing the lowest percentage (5%). The eluent from each washing step was collected, evaporated to dryness and reconstituted with the mobile phase prior to analysis for diazepam using the LC-MS-MS method. The experiment was carried out in triplicate.

It was found that the last traces of diazepam template were removed in the washing step using 10 % acetic acid in acetonitrile (Figure 6-7). Therefore, 15% acetic acid in acetonitrile was used as the eluent in all subsequent elution steps.

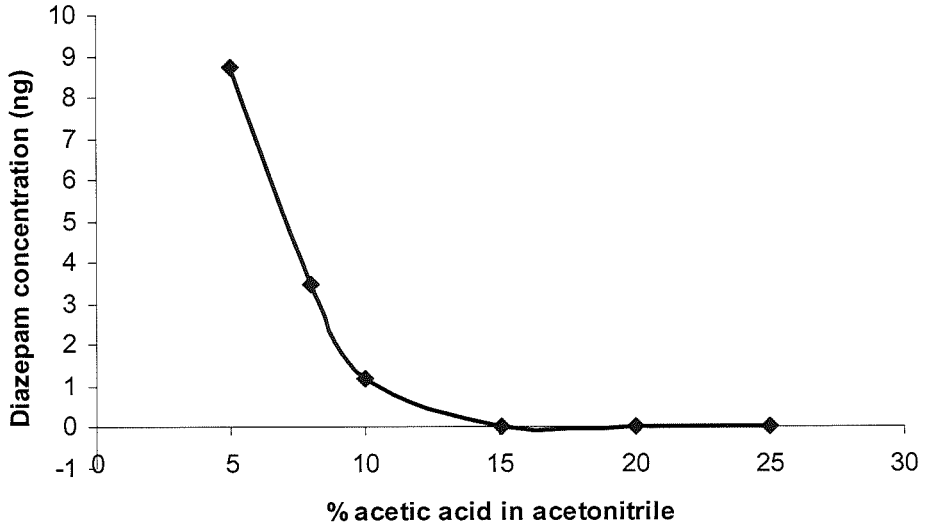


Figure 6-7 Effect of eluent on diazepam bindings.

6.4.10.2 Effect of the solvent on diazepam binding to imprinted and non-imprinted polymers

The solvent plays an important role in the recognition step. For those cases where recognition is driven by hydrogen bonding, the interaction forces between the analyte and the polymer are diminished when increasingly polar solvents are applied. To determine the effect of solvent on the diazepam MIP, the imprinted and non-imprinted polymers were conditioned with 0.5 mL chloroform, dichloromethane and toluene prior to loading of 1 mL of 50 ng diazepam in each solvent, respectively. The cartridges were then washed with 0.5 mL of the same solvent used in the loading step, before eluting with 0.5 mL 15% (v/v) acetic acid in acetonitrile. The fractions collected after the loading (FL), washing (FW) and elution (FE) steps were blown down using a stream of nitrogen gas and reconstituted in 100 µl of the LC-MS-MS mobile phase, 20 µl of which was injected for analysis. The percentage of diazepam bound to the polymer was calculated using Equation 6-1.

Equation 6-1

% bound = (peak area for FE/peak area of (FL + FW + FE)) x 100

The percentage of diazepam bound to the polymers using three different solvents in the extraction protocol is shown in Figure 6-8. This data enables the optimal solvent for further experiments to be identified. In theory, MIPs should exhibit optimal molecular recognition when in the presence of the solvent that was used as a porogen during polymerization; in the present case, this was chloroform. However, the percentage of diazepam bound is lower when using chloroform than for toluene, suggesting that tighter binding is gained in the more apolar solvent. In other words, a solvent with lower dielectric constant (ϵ) is a more effective solvent for rebinding. Therefore, toluene was chosen as a loading solvent in the study.

The lowest percentage of diazepam bound to the MIP was observed when loading and washing with acetonitrile. Acetonitrile, with a higher hydrogen bonding donor parameter interefered with the binding between the substrate (diazepam) and the MIP. Therefore, leading to decreased binding affinity of the substrate to the MIP was decreased.

Nonspecific binding of diazepam to the non-imprinted polymer is to be anticipated because of lipophilic interactions between the analyte and the organic polymer structure. The tests conducted also indicated that this type of non-specific interaction was minimised when toluene was the solvent. Just as a hydroxylic solvent is expected to interfere with hydrogen bonding between analyte and sorbent, a lipophilic solvent would be expected to interfere with lipophilic interactions.

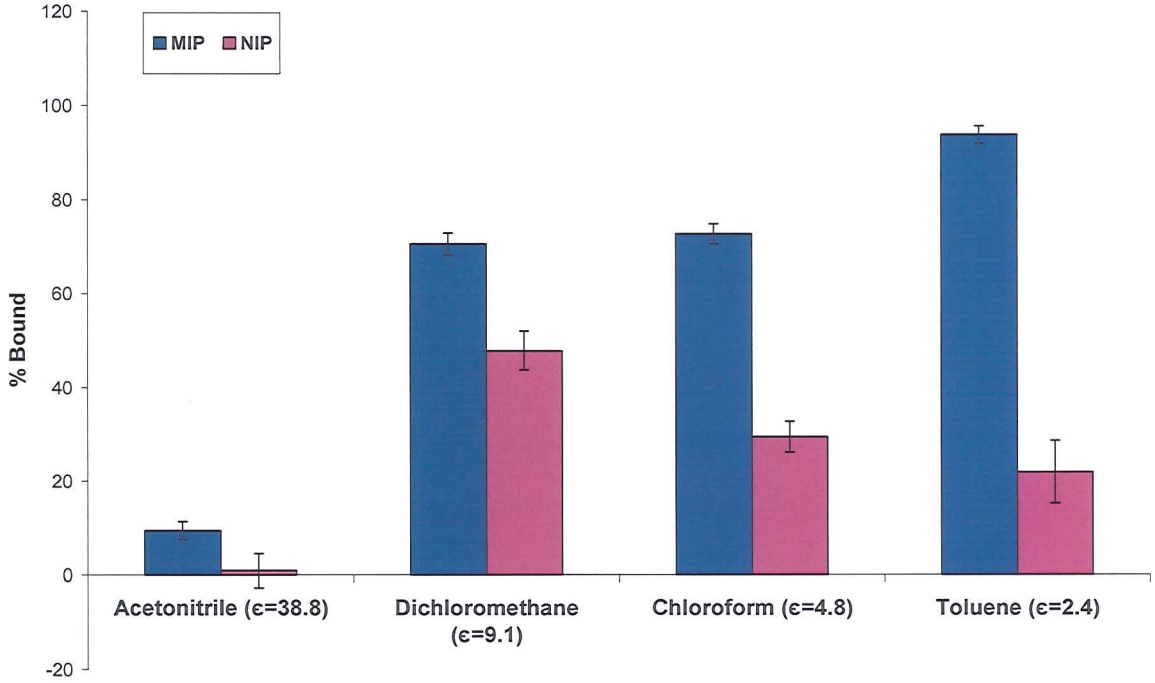


Figure 6-8 Mean percentage of diazepam bound to the MIP and NIP using dichloromethane, chloroform and toluene as loading solvents (n=3). Data expressed as the mean ± standard deviation.

To optimize the extraction protocol, different volumes of the washing solvent were evaluated. The extraction was also carried out with 0.5 mL of 0.5, 1 and 2% of acetic acid in toluene. From the results shown in Figure 6-9, it was found that washing with acetic acid in toluene resulted in the loss of most of the analyte from the cartridge. Washing with pure toluene allowed most of the analyte to be recovered. A volume of 0.3 mL of toluene was sufficient to deliver cleaner extracts without a significant loss of diazepam during the extraction process.

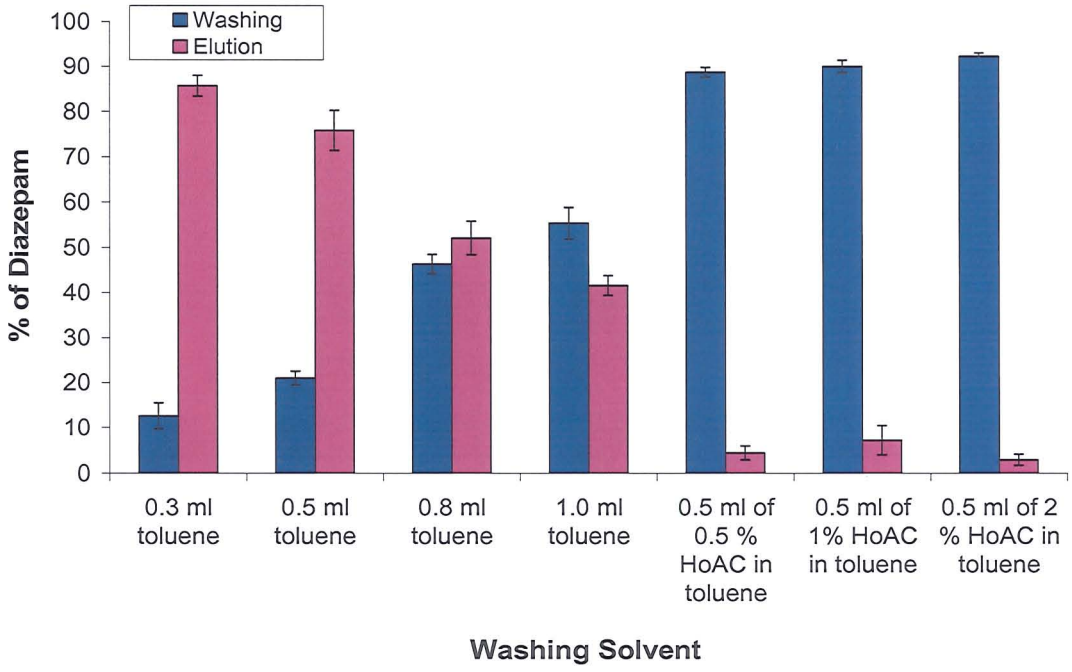


Figure 6-9 Mean recovery of diazepam in the washing and elution fractions after loading 1.0 mL of 50 ng/mL diazepam prepared in toluene, by using different washing solutions (n=3). Elution solvent: 0.5 mL 15% acetic acid in acetonitrile. Data expressed as the mean ± standard deviation.

6.4.11 Evaluation and validation of diazepam MISPE for hair samples

The evaluation and validation of a method for diazepam analysis in hair samples followed the optimized MISPE protocol summarized in Table 6-3.

Table 6-3 Optimized MISPE protocol

Step	Solvent
Loading	Pre-extract from hair was evaporated to dryness and reconstituted with 1 mL toluene (loading solvent).
Conditioning	0.5 mL 15% of acetic acid in acetonitrile, 0.5 mL toluene
Washing	0.3 mL toluene
Elution	0.5 mL 15% of acetic acid in acetonitrile

6.4.11.1 Binding capacity of diazepam MIP

Determination of the diazepam binding capacity for the MIP cartridge was performed using the optimized procedure, with toluene as the solvent of choice. Different quantities of diazepam ranging from 500-2600 ng were dissolved in 1 mL toluene and extracted separately using different diazepam MIP cartridges. 100 ng internal standard was added to the eluent after the extraction. The eluent was evaporated to dryness with a nitrogen stream and the residue was reconstituted with the initial mobile phase prior to analysis by LC-MS-MS.

6.4.11.2 Cross-reactivity of diazepam MIP with morphine

It has been postulated that the selective molecular recognition within the molecularly imprinted polymer cavity can be attributed not only to cooperative binding of pre-organized functional groups but also to the shape selectivity that is complementary to the template [147].

In this study, morphine was selected to investigate the role of shape selectivity in diazepam MIP. 50 ng morphine was subjected to the same extraction procedure as above in triplicate. The eluent collected from the extraction and two unextracted samples containing the same concentration of morphine were blown down and reconstituted in the mobile phase prior to LC-MS-MS analysis. The percentage of morphine bound to the diazepam MIP was calculated using Equation 6-2.

Equation 6-2

% bound = (peak area of extracted morphine/ peak area of unextracted morphine) x 100

6.4.11.3 Recoveries of diazepam and other benzodiazepine derivative drugs

Benzodiazepine derivative drugs, namely 7-aminoflunitrazepam, oxazepam, lorazepam, chlordiazepoxide, temazepam, flunitrazepam, nordiazepam, nitrazepam and diazepam, were used to probe the group selectivity of the diazepam MIP. 50 ng samples of each drug were prepared in 1 mL toluene (n=5). The optimized extraction protocol was used to determine the recoveries of benzodiazepines with the MIP and NIP. The cartridge

was preconditioned with 0.5 mL of toluene. Each sample was passed through a cartridge and washed with 0.3 mL toluene. The analytes retained in the cartridge were eluted with 0.5 mL 15% (v/v) acetic acid in acetonitrile. 100 ng of internal standard was then added after the extraction.

The recovery was calculated by comparing peak area ratios obtained from extracted samples (quantitation ion/internal standard) to the peak area ratios of the same concentration of pure standards (quantitation ion/internal standard) in unextracted samples. The percent mean recovery and percent relative standard deviation (RSD) was calculated for each drug at each concentration.

6.4.11.4 Limits of Detection (LOD) and Lower Limits of Quantification (LLOQ)

The LOD and LLOQ were determined for each drug using spiked hair. 30 mg of blank, decontaminated hair was spiked with 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 2 and 5 ng of each drug and 100 ng of the internal standard mixture to produce a regression line for each analyte (n=3). The samples were then pre-extracted, extracted with MISPE and analyzed by LC-MS-MS. The LOD and LLOQ were determined as signal-to-noise ratios of 3 and 5, respectively.

6.5 Results and Discussion

6.5.1 Binding capacity of diazepam MIP

Peak area ratios from the LC-MS/MS analysis of diazepam recovered by MISPE were plotted against the diazepam amount loaded on the cartridge to determine the maximum binding capacity value and are depicted in Figure 6-10. From the graph, the amount of diazepam recovered is constant above a loading of approximately 2200 ng. The cartridge contained 20 mg MIP so the binding capacity of the imprinted polymer was determined to be 110 ng of diazepam per mg of polymer, which is lower than the range reported from previous studies [102, 146]. Higher binding capacities were found in MIPs prepared using acetonitrile as a porogen. Acetonitrile is believed to create a more porous polymer which leads to better access for the analyte through the polymer monolith [148].

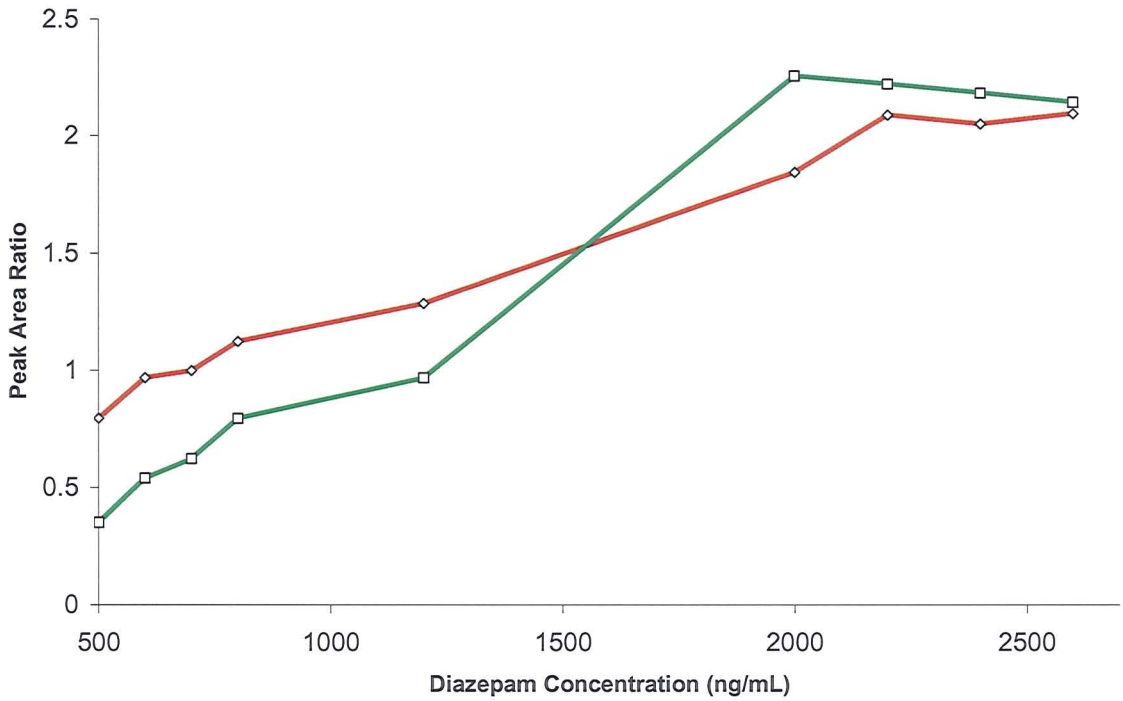


Figure 6-10 Graph plots from two separate experiments to estimate the binding capacity value using MIP cartridges.

6.5.2 Cross-reactivity of diazepam MIP with morphine

By using the same extraction protocol for morphine as was used for diazepam, the cross-selectivity of the MIP for morphine was found to be less than 15%. This finding indicates that a substrate with a different shape and different functional groups from those of the template has lower affinity towards the MIP, hence the MIP has decreased selectivity towards this substrate. Similar findings were observed in other cross-reactivity studies on MIPs [147, 149].

6.5.3 Recoveries of diazepam and other benzodiazepine derivative drugs

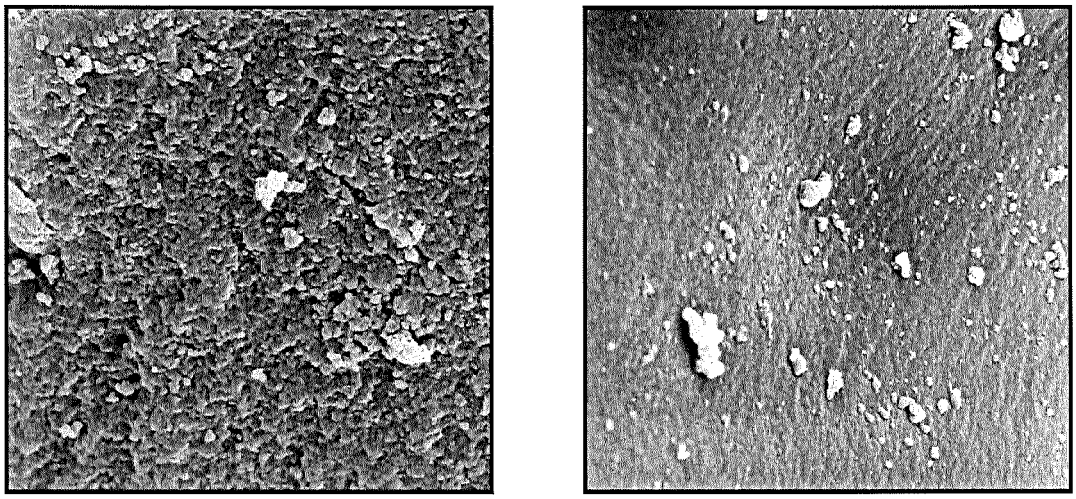
The results showed that the MIP recovered a high percentage of 7-aminoflunitrazepam, lorazepam, temazepam, nordiazepam, nitrazepam and diazepam, with acceptable percent RSD values (Table 6-4). Flunitrazepam and chlordiazepoxide were bound to a lesser extent presumably due to a poorer fit in the imprinted binding pockets.

Table 6-4. Recoveries of benzodiazepines on the MIP and NIP

Analyte	Mean % Recovery (% RSD) for n=5, Amount added = 50 ng/30 mg hair	
	MIP	NIP
7-Aminoflunitrazepam	91.9 (1.5)	71.0 (7.5)
Oxazepam	73.4 (8.7)	41.4 (34.3)
Lorazepam	97.2 (17.0)	48.1 (22.0)
Chlordiazepoxide	61.6 (13.5)	32.0 (86.8)
Temazepam	89.6 (13.4)	54.3 (52.9)
Flunitrazepam	39.0 (5.1)	2.3 (67.8)
Nordiazepam	102.9 (9.8)	65.0 (14.3)
Nitrazepam	92.3 (5.42)	60.6 (33.9)
Diazepam	93.0 (1.5)	16.3 (17.1)

The high recovery of diazepam from the MIP resulted from the excellent molecular recognition of the template molecule imparted by the imprinting process. Lower recoveries were obtained when the NIP was used as the extraction sorbent, as expected, together with high percent RSD values. Nevertheless, it was noted that high recoveries were obtained for some drugs with the NIP, suggesting that perhaps not all of the drugs were being selectively adsorbed on the MIP. The high RSD values may reflect the fact that the NIP is ill-defined in respect to its binding character compared to the MIP.

The surface morphology for both MIP and NIP was determined with the scanning electron microscope (SEM) and is depicted in Figure 6-11. The images show that the NIP had a smoother and more uniform surface compared to the MIP. Rough and irregular shapes present on the MIP surface were probably due to the fact that the specific binding sites have been created and probably caused by the structure of the template [148].



(a) (b)

Figure 6-11 SEM micrographs of (a) diazepam MIP and (b) NIP (x 10,000).

6.5.4 Calibration Model, Limits of Detection (LOD) and Lower Limits of Quantification (LLOQ)

The linear correlation coefficients (r^2) of the calibration curves were > 0.99 for all analytes, except for flunitrazepam, for which r^2 was 0.98 (n=3). Calibration data for the benzodiazepines are summarised in Table 6-5.

Table 6-5 Calibration data for the benzodiazepines

Analyte	r^2	b	a
7-Aminoflunitrazepam	0.9980	0.0004	-0.00004
Oxazepam	0.9994	0.0022	0.00007
Lorazepam	0.9900	0.0023	0.0004
Chlordiazepoxide	0.9968	0.0012	0.0004
Temazepam	0.9965	0.0037	-0.0013
Flunitrazepam	0.9895	0.00001	0.00002
Nordiazepam	0.9983	0.0007	-0.00009
Nitrazepam	0.9999	0.0023	0.00006
Diazepam	0.9980	0.0004	-0.00004

Typical equation is $y = bx + a$, where x is the analyte concentration and y is the peak area ratio of analyte and internal standard.

The LOD and LLOQ for benzodiazepines ranged from 0.03 to 0.78 ng/mg and from 0.06 to 1.32 ng/mg using 30 mg hair, respectively (Table 6-6).

Table 6-6 The LOD and LOQ values for the benzodiazepines

Analyte	LOD (ng/mg)	LOQ (ng/mg)
7-Aminoflunitrazepam	0.03	0.06
Oxazepam	0.13	0.21
Lorazepam	0.66	1.11
Chlordiazepoxide	0.33	0.57
Temazepam	0.39	0.63
Flunitrazepam	0.78	1.32
Nordiazepam	0.21	0.33
Nitrazepam	0.06	0.11
Diazepam	0.09	0.14

Specimen chromatograms of quantitation ions for all benzodiazepines at 2 ng/mg and blank extracts are shown in Figure 6-12. The extracts from the hair samples using MISPE were clean and free from interferences. The blank extract chromatogram shows no diazepam peak, clearly indicating that no bleeding of templates was observed. Even if template bleeding were to be observed, then the group-selective binding character of the MIP would still allow for the accurate quantitation of diazepam metabolites and other members of the benzodiazepine family even if diazepam itself could not be analysed.

The LOD and recovery values for diazepam by MISPE were 0.09 ng/mg hair and 93%, respectively, compared to 0.13 ng/ mg hair and 69% by the conventional SPE method described by Miller *et al.* which used the same pre-extraction method and LC-MS analysis [143]. Chapter 7 in this thesis considers a comparison of MISPE and SPE for benzodiazepines in hair. However the sensitivity of this method is still not as good as obtained in other published work using a more sensitive detector [136, 138, 139].

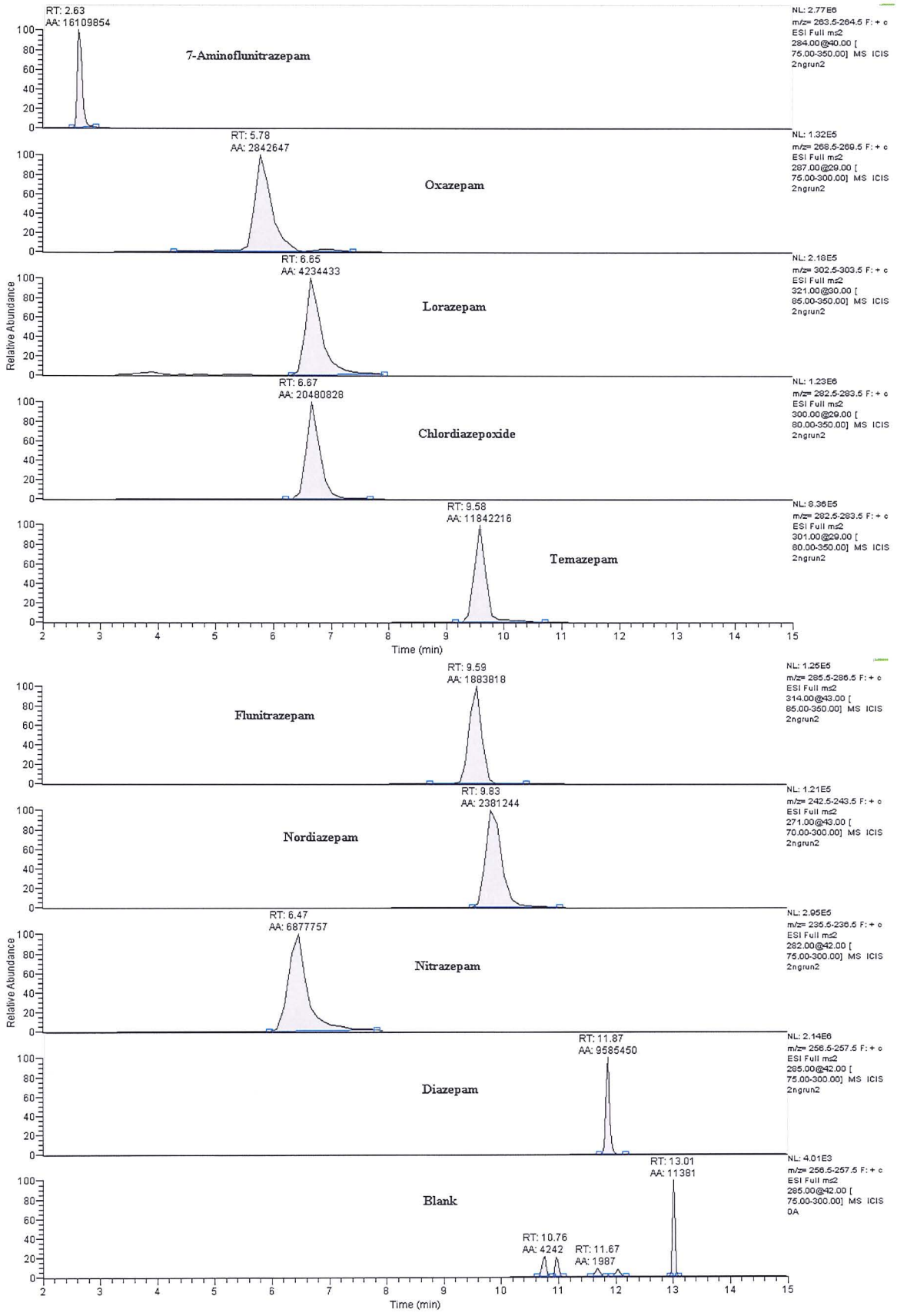


Figure 6-12 Chromatograms of quantitation ions of benzodiazepines in blank hair spiked at a concentration of 2 ng, and a blank hair extract, subsequent to the MISPE step.

6.6 Conclusion

In this study, an MIP extraction method for diazepam in hair samples has been developed. Results for diazepam analysis have been found to be accurate, reproducible and sensitive. The method also works for other benzodiazepine drugs due to the group-selective binding nature of the MIP. The performance characteristics, in terms of sensitivity and selectivity, indicate that this method is sufficiently accurate and precise to be used for trace diazepam analysis in hair samples, a finding that may be of particular relevance to the investigation of drug-facilitated crime cases.

7 COMPARISON OF MISPE WITH CONVENTIONAL SPE FOR THE DETECTION OF BENZODIAZEPINES IN POST-MORTEM HAIR SAMPLES

7.1 Introduction

SPE methods have been well validated and accepted for extraction of benzodiazepines from hair samples prior to LC-MS-MS analysis [143]. Previously, as described in Chapter 6, an anti-diazepam MIP was synthesized and its application to MISPE was validated. It showed better selectivity towards the template, diazepam, and its metabolites as well as for the other benzodiazepines tested. To confirm the findings, a comparison between MISPE and SPE performance was necessary and appropriate.

7.2 Aim

The aim of this study was to compare the benzodiazepine hair results for ten post-mortem scalp hair case samples using a conventional SPE system and the MISPE system developed previously and described in Chapter 5. The study would determine if MISPE showed any advantages over conventional SPE in terms of improved sensitivity and specificity and if the number of potential interferences was reduced.

7.3 Experimental

7.3.1 Instrumentation

LC-MS-MS analysis of benzodiazepines was carried out using a Thermo Finnigan LCQ Deca XP (Thermo Finnigan, San Jose CA, USA) equipped with a Surveyor autosampler and MS pump system. Chromatographic separation was performed using a Gemini C18 column (150 mm x 2.0 mm ID, 5 µm particle size) and a 4.0 mm x 2.0 mm, 5 µm guard column (Phenomenex, Torrance CA, USA) with the same packing as the column.

7.3.2 Chemicals and Materials

Diazepam for polymer synthesis was obtained from Roche (Hertfordshire, UK). Methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) were from

Aldrich (Steinhem, Germany), chloroform was from Rathburn (Peebles, UK) and 2,2'-azobisisobutyronitrile (AIBN) was from Acros Organics (Geel, Belgium).

Ammonium formate and sodium dodecyl sulphate were from Sigma-Aldrich (Dorset, UK). HPLC grade acetonitrile, formic acid, toluene, chloroform and dichloromethane were purchased from BDH (Poole, UK).

Empty 1mL polyethylene SPE cartridges and frits (20 μm mean pore size) for diazepam MISPE were purchased from International Sorbent Technology Limited (Mid Glamorgan, UK). World Wide Monitoring Clean Screen® columns (ZSDAU 020) were purchased from United Chemical Technologies, Inc. (Pennsylvania, USA).

7.3.3 Standards

For LC-MS-MS analysis, diazepam, nordiazepam, nitrazepam, chlordiazepoxide, temazepam, oxazepam, lorazepam, flunitrazepam and 7-aminoflunitrazepam and the internal standards diazepam-d5, nordiazepam-d5, temazepam-d5, oxazepam-d5, lorazepam-d4, flunitrazepam-d7 and 7-aminoflunitrazepam-d7 were purchased from Promochem (Teddington, UK).

7.3.4 Preparation of Solutions

7.3.4.1 Mobile Phase

A mobile phase consisting of 3 mM ammonium formate and 0.001% formic acid in water was prepared by adding 0.189 g ammonium formate and 10 μl of concentrated formic acid to a 500 mL volumetric flask and making up to 500 mL with deionised water.

7.3.4.2 Preparation of pH 6.0 Phosphate Buffer

1.70 g of sodium hydrogen phosphate and 12.14 g of sodium dihydrogen phosphate were weighed into a 1 L volumetric flask and made up to volume with deionised water. The pH was adjusted to pH 6.0 using phosphoric acid.

7.3.5 Post-mortem Samples

The ten hair samples tested were post-mortem case samples submitted to the toxicology laboratory of Forensic Medicine and Science, University of Glasgow. Analysis of case materials was carried out under a protocol agreed with the Procurators Fiscal by Professor J.S. Oliver and which is followed by all research students in the Section, such that results of analysis should be given to the Procurators for use in the case investigation. One scalp hair sample was collected in each case. The case samples selected for testing were cases in which the blood result was positive for benzodiazepines using a validated in-house LC-MS-MS method [150]. Eleven negative hair samples were obtained from volunteers and post-mortem case samples. All samples were wrapped in aluminium foil and stored at room temperature prior to analysis.

7.3.6 Preparation of Hair Samples

The root-0.5 cm section was removed from each hair case sample where roots were present. The 0.5 cm-tip section was weighed out into a vial for analysis and cut into 2-3 mm segments using a pair of clean scissors. The hair case samples were washed with 1 mL of 0.1% sodium dodecyl sulfate, 2 x 1 mL deionised water and 2 x 1 mL dichloromethane, each with a 10 minute sonication. The likelihood of benzodiazepine contamination is very low, therefore analysis of washings as performed in the laboratory during validation of the method was not necessary [143]. Each sample was then split between two separate vials for extraction by SPE and MIP procedures. Blank hair was also washed using the same procedure prior to spiking with benzodiazepine standards to produce the calibrators.

1.5 mL of a solution containing methanol and 25% ammonium hydroxide solution (20:1 v/v) was added to each calibrator and sample, and the suspension was left to sonicate for one hour and then stored at room temperature overnight. The solvent was then removed from the vials and transferred to a test tube. The hair was washed twice with 0.75 mL of the same solvent mixture and the washings were removed and transferred to a test tube. The contents of the test tubes were evaporated to dryness under a stream of nitrogen and the residues were reconstituted in 1 mL of phosphate buffer (0.1 M, pH 6.0) for extraction by the validated conventional SPE method or in 1 mL toluene for extraction by the diazepam MISPE method.

7.3.6.1 Conventional SPE Method

The conventional solid-phase extraction method selected for this study operated by a mixed-mode cationic exchange mechanism and is based on work reported by Miller *et al.* [143]. The sorbent is composed of C8 chains and benzene sulfonic acid (BSA) residues. Benzodiazepines are retained on the column *via* both hydrophobic and ionic interactions.

World Wide Monitoring Clean Screen® columns (ZSDAU 020) were conditioned sequentially with 3 mL methanol, 3 mL distilled water and 1 mL of phosphate buffer (0.1M, pH 6.0). The vortexed samples were loaded onto the columns and allowed to drip through without the presence of a vacuum. The columns were washed sequentially with 2 mL distilled water, 2 mL 20% acetonitrile in phosphate buffer (0.1 M, pH 6.0), 2 mL cyclohexane and 2 mL distilled water. The columns were dried under full vacuum after the second, third and final parts of the wash sequence, for 5, 1 and 5 minutes respectively. The analytes were eluted using 1.5 mL 2% ammoniated ethyl acetate (followed by a 2 minute drying step on full vacuum) and 1.5 mL dichloromethane:isopropanol:ammonium hydroxide (78:20:2). The eluted samples were blown down to dryness under a stream of nitrogen and reconstituted in 100 µL of the mobile phase in its initial composition. 20 µL of the reconstituted extract was injected for analysis.

7.3.6.2 Diazepam MIP Extraction Method

Diazepam MIP cartridges were prepared as detailed in Section 6.4.6. The cartridges were conditioned using 0.5 mL of toluene. The vortexed samples were added to the cartridges and allowed to drip through without the presence of a vacuum. The columns were washed with 0.3 mL of toluene and analytes were then eluted using 0.5 mL 15% (v/v) acetic acid in acetonitrile. The eluted samples were blown down to dryness under a stream of nitrogen and reconstituted in 100 µL of the mobile phase in its initial composition. 20 µL was injected for analysis.

7.3.7 LC-MS-MS Conditions

LC was carried out using a mobile phase containing 3 mM ammonium formate and acetonitrile at a flow rate of 0.3 mL min^{-1}. The elution program consisted of a linear gradient (65-20%) of 3 mM ammonium formate over 13 minutes. The percentage of

ammonium formate was then decreased to 10% between 13 to 13.5 minutes. It was held at 10% between 13.5 to 16.5 minutes before being increased to the initial condition (65%) between 16.5 to 20 minutes. 20 µl samples were injected on the Gemini C18 column.

All mass spectral data was acquired in electrospray positive ion mode. The capillary temperature, sheath and auxiliary gas flow rates and collision energies were optimized for each analyte. The probe voltage used was 4.5 kV. Internal standard data was collected in selected ion monitoring (SIM) mode and analytes were identified on the basis of their retention time and full MS-MS spectra. The product ion ratios were monitored to gain further qualitative identification data. The optimum tuning parameters, precursor and product quantitation ions are shown in Table 6-2.

7.3.8 Results

Hair sample weights, color and section length analyzed are summarised in Table 7-1. The concentrations of detected benzodiazepines in hair samples using both SPE and MIP methods, and in blood samples using validated in-house SPE results are shown in Table 7-2.

Table 7-1 Hair sample weights, colour and section length analysed

Case Sample Number	Weight SPE (mg)	Weight MISPE (mg)	Hair Colour	Section Length (cm)
1	9.88	9.91	Dark brown	0.5-11.5
2	9.29	9.15	Dark brown	0.5-3
3	9.35	9.87	Light brown	0.5-2.5
4	10.08	9.96	Blonde	0.5-12
5	9.90	9.91	Brown	0.5-4.5
6	9.86	10.05	Dark brown	0.5-3
7	9.95	9.99	Dark brown	0.5-6
8	10.02	10.12	Dark brown/black	0.5-7
9	9.98	9.89	Brown	0.5-7
10	9.80	9.10	Black	0.5-3.5

Table 7-2 SPE and MISPE benzodiazepine hair results *vs.* blood analysis results

Case	Diazepam			Nordiazepam			Oxazepam			Temazepam			Nitrazepam		
Sample Number	Hair (ng/mg)		Blood (mg/L)	Hair (ng/mg)		Blood (mg/L)	Hair (ng/mg)		Blood (mg/L)	Hair (ng/mg)		Blood (mg/L)	Hair (ng/mg)		Blood (mg/L)
	MISPE	SPE		MISPE	SPE		MISPE	SPE		MISPE	SPE		MISPE	SPE	
1	0.06	-	0.03	0.09	0.28	0.01	-	-	-	-	0.22	-	-	-	-
2	0.68	0.65	0.72	0.97	1.20	1.42	0.06	-	0.08	0.17	0.24	0.13	0.23	0.24	-
3	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-
4	-	-	0.03	0.08	-	0.05	-	-	-	-	-	-	-	-	-
5	0.03	-	0.15	0.08	0.27	0.2	-	-	0.02	-	-	0.02	-	-	-
6	0.46	0.46	0.11	-	-	-	-	-	-	-	-	-	-	-	-
7	0.06	-	0.04	0.19	0.31	0.08	-	-	-	0.16	0.22	-	-	-	-
8	0.08	0.03	0.02	0.12	0.43	0.04	-	-	-	-	-	-	-	-	-
9	0.02	-	0.15	-	-	0.07	-	-	-	-	-	0.01	-	-	-
10	0.50	0.06	0.06	0.12	0.28	0.16	0.09	0.59	-	-	0.23	-	-	-	-

'-' = not detected

Benzodiazepines were detected in nine case samples using the MIP extraction method and seven case samples using the conventional SPE method. Diazepam was detected in four samples using the MIP extraction where there was no diazepam detected using the SPE method.

Nordiazepam was detected in Sample 4 using the MIP extraction where a very low blood level was detected and was not detected in this sample using SPE. In Sample 3, no benzodiazepines were detected where there was a very low nordiazepam blood level. This finding is consistent with another study which found that one hair sample tested negative for nordiazepam where the blood result was 0.20 mg/L [141].

Temazepam was detected in Samples 4 and 10 by SPE where none was detected by MIP extraction. It appeared from the results that no temazepam was detected in hair when there were very low blood levels. Oxazepam was detected in Sample 2, which had a very low blood concentration, using the MIP extraction where none was detected by SPE. In contrast, a higher oxazepam concentration was detected in another sample using SPE than the concentration which was detected using MISPE. The nitrazepam results for SPE and MISPE are in good agreement.

7.4 Discussion

In this study, benzodiazepine concentrations were measured in ten post-mortem scalp hair samples using SPE and MIP extraction methods. The overall results show the most frequently found drug in the hair of the cases studied was diazepam, followed by its metabolite nordiazepam. In general, benzodiazepine levels were in the ranges reported in the literature.

Nordiazepam was always detected at a higher concentration than diazepam, a finding which is consistent with other studies [133,141]. Oxazepam was only detected in two samples. The relatively short half life of oxazepam compared to nordiazepam and the fact that it is a polar compound which may not be readily incorporated into hair might explain this low detection rate [131]. In addition to this the oxazepam blood levels were also very low.

Temazepam levels in hair have not been widely reported. However, in this study, temazepam levels were detected even though within the relatively narrow range of 0.16-

0.24 ng/mg. It is possible that temazepam was the parent drug ingested in the two temazepam positive samples. In the two case samples that tested negative, where the blood levels were positive at very low levels, temazepam may have been present as a metabolite of diazepam, rather than the parent drug.

The nitrazepam concentration detected in one case sample by MIP and SPE was present at a higher level than reported in another study [117]. Overall, it is difficult to compare directly the benzodiazepine levels detected in our study with those reported by other authors because of differences in the extraction and analytical methods used. It is also difficult to interpret the benzodiazepine levels detected in the hair since the amount and type of drug taken over the months prior to death is not known in the majority of cases. As well as potentially being present in hair samples due to diazepam metabolism, nordiazepam, temazepam and oxazepam could also be ingested as parent drugs or they could be present in association with both metabolism and parent drug use.

The police reports for the cases provided some information on the samples. Hair sample 6 came from an individual who had been prescribed Valium® in the past; hair sample 8 belonged to an individual who had previously overdosed on Valium® and ecstasy, and hair sample 10 was from an individual who had been prescribed diazepam.

Examples of the chromatograms for blank hair standard, lowest diazepam standard, diazepam positive and negative samples are shown in Figure 7-1. In the samples where both extraction methods tested positive for diazepam, the concentrations detected using the MIP extraction were always higher than those obtained using the SPE method. The higher recovery using the MIP extraction might be due to the excellent molecular recognition of the template molecule and the lower LOD and LOQ.

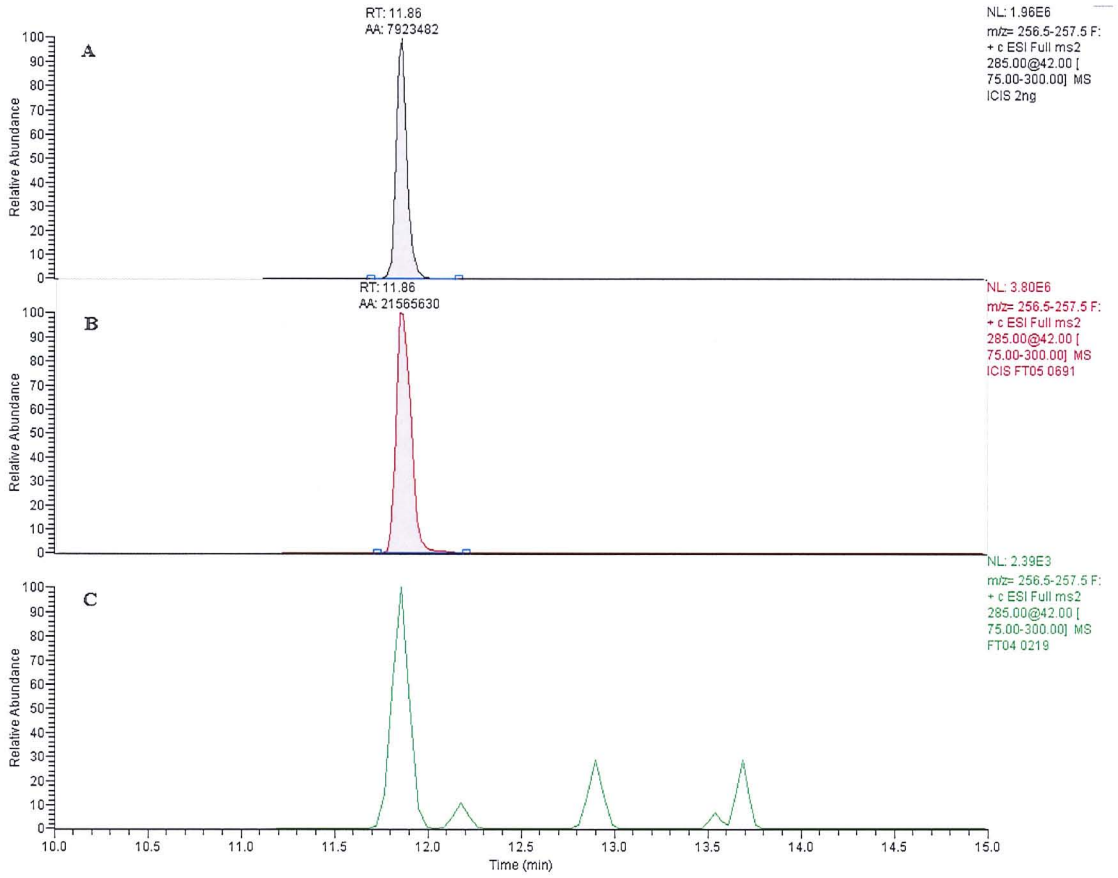


Figure 7-1 Chromatogram of A) blank hair spiked at 2 ng diazepam, B) diazepam positive case sample and C) diazepam negative case sample.

Table 7-3 Recoveries and LOD values of benzodiazepines using MISPE and SPE methods

Analyte	Mean % Recovery (% RSD) for n=5 Amount added = 50 ng/mL		LOD (ng/mg)	
	MISPE	SPE	MISPE	SPE
7-Aminoflunitrazepam	92 (2)	55 (10)	0.03	0. 14
Oxazepam	73 (9)	83 (8)	0.13	0.11
Lorazepam	97 (17)	94 (10)	0.66	0.62
Chlordiazepoxide	62 (14)	63 (5)	0.33	0.07
Temazepam	90 (13)	88 (8)	0.39	0.09
Flunitrazepam	39 (5)	89 (6)	0.78	0.30
Nordiazepam	103(10)	82 (5)	0.21	0.24
Nitrazepam	92 (5)	91 (9)	0.06	0.03
Diazepam	93 (2)	69 (2)	0.09	0.13

In the samples where both extraction methods tested positive for nordiazepam, temazepam and oxazepam, the concentrations detected were always higher using the SPE method. The extraction recoveries for these analytes were higher using the MIP method and while the LOD and LOQ for nordiazepam and oxazepam were similar for both extraction methods, they were higher for temazepam using MISPE. The higher temazepam LOD and LOQ for the MIP extraction could explain the lower concentrations detected, compared to SPE. Nitrazepam levels detected by MISPE and SPE were very similar. Despite the lower concentrations detected by the MIP extraction, the results showed that the method was selective for metabolites and other benzodiazepine drugs and that it detected diazepam and oxazepam in samples which tested negative using SPE.

Using MISPE, the extracts for diazepam and nordiazepam were observed to be cleaner than SPE extracts, showing less LC-MS-MS interference (Figure 7-2). An interfering peak was found in the SPE extract during the diazepam analysis at a retention time (t_R) of 12.70 min but none was present in the MISPE extract. Interference was also found in the SPE extract during the nordiazepam analysis at a t_R of 10.84 min and again this was not present in the MISPE extract.

7.5 Conclusion

The results of this preliminary study strongly suggested that the diazepam MIP can be successfully applied for the detection of benzodiazepines in post-mortem hair case samples. Due to the higher extraction recovery using MIP and excellent recognition of the template molecule, the MIP procedure detected a higher number of diazepam positive case samples compared to the SPE procedure. This method has also demonstrated selectivity for other benzodiazepine drugs and detected oxazepam in one sample where SPE detected none. However it is not as selective for temazepam as the SPE method. Nordiazepam, oxazepam and temazepam concentrations when detected using both extraction methods, were higher using the SPE method. Further work is needed to determine if the MIP cartridges could be used to detect benzodiazepines in single dose samples.

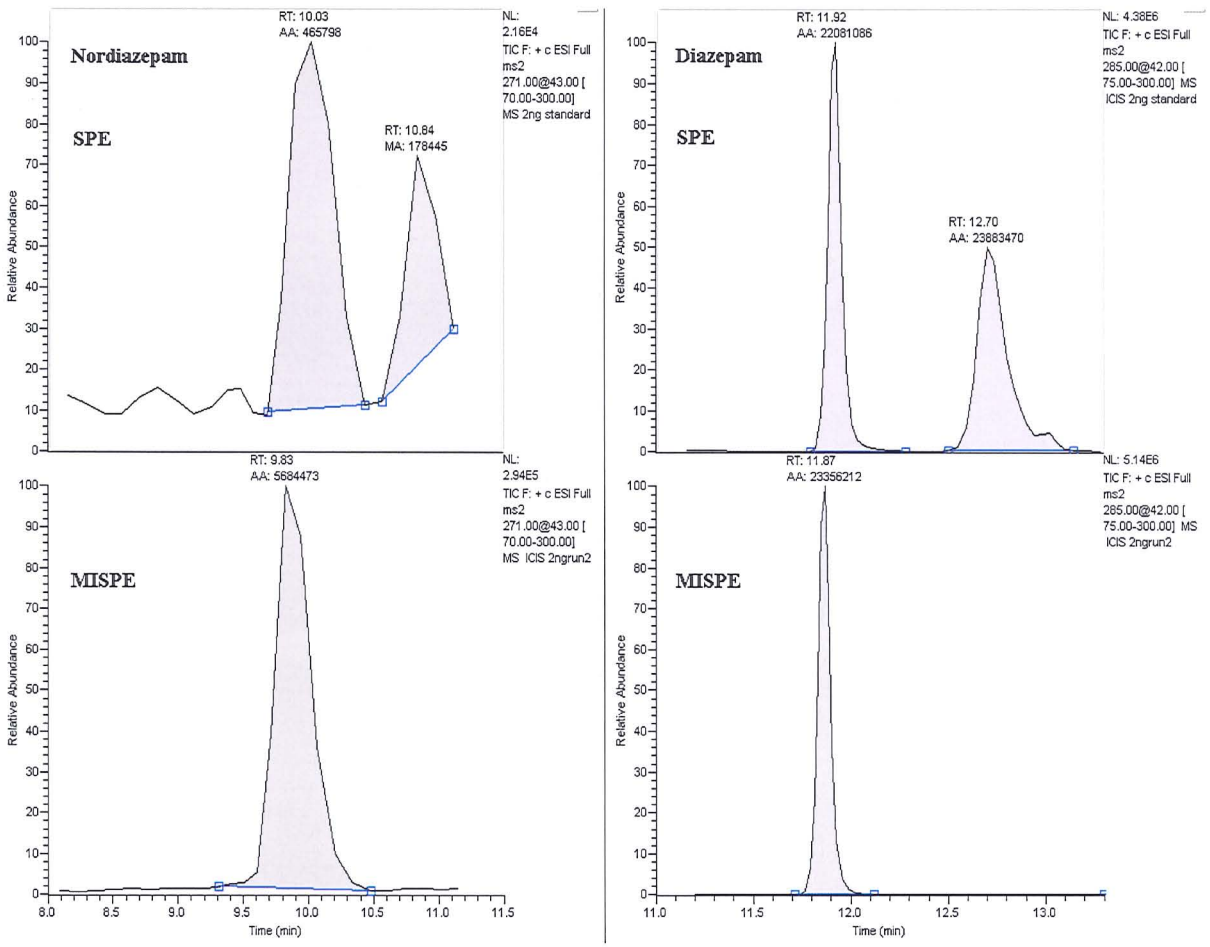


Figure 7-2 Chromatograms of the SPE and MISPE extracts showing LC-MS-MS interference in the nordiazepam and diazepam scan segment for the SPE extract only.

8 MOLECULARLY IMPRINTED SOLID-PHASE EXTRACTION OF FLUNITRAZEPAM

8.1 Introduction

In Chapter 6, an-anti diazepam MIP was found to be a good sorbent for the extraction of diazepam and other benzodiazepine derivative drugs from hair samples. However, benzodiazepines which have a larger molecular size compared to diazepam (template) such as flunitrazepam and chlordiazepoxide displayed a lower binding affinity toward the MIP. Similar findings were observed in previous studies [151, 152]. It has been reported that a smaller molecule should be able to bind in a larger binding site as long as it possesses the same functional site interactions, but not *vice versa* due to steric hindrance effects.

8.2 Aim

The aim of this study was to synthesise an MIP using flunitrazepam as a template and apply it to MISPE by using the same protocol developed for diazepam MISPE. The performance of the flunitrazepam MIP would then be compared to the diazepam MIP.

8.3 Experimental

8.3.1 Instrumentation

LC-MS-MS analysis of benzodiazepines was carried out using a Thermo Finnigan LCQ Deca XP (Thermo Finnigan, San Jose CA, USA) equipped with a Surveyor autosampler and MS pump system. Chromatographic separation was performed using a Gemini C18 column (150 mm x 2.0 mm ID, 5 μm particle size) and a 4.0 mm x 2.0 mm, 5 μm guard column (Phenomenex, Torrance CA, USA) with the same packing as the column.

8.3.2 Chemicals and Materials

Flunitrazepam for polymer synthesis was obtained from Roche (Hertfordshire, UK). Methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) were from Aldrich (Steinhem, Germany), chloroform was from Rathburn (Peebleshire, UK) and 2,2'-azobisisobutyronitrile (AIBN) was from Acros Organics (Geel, Belgium).

Ammonium formate and sodium dodecyl sulphate were from Sigma-Aldrich (Dorset, UK). HPLC grade acetonitrile, formic acid, toluene, chloroform and dichloromethane were purchased from BDH (Poole, UK).

Empty 1mL polyethylene SPE cartridges and frits (20 μm mean pore size) were purchased from International Sorbent Technology Limited (Mid Glamorgan, UK).

8.3.3 Standards

For LC-MS-MS analysis, diazepam, nordiazepam, nitrazepam, chlordiazepoxide, temazepam, oxazepam, lorazepam, flunitrazepam and 7-aminoflunitrazepam and the internal standards diazepam-d5, nordiazepam-d5, temazepam-d5, oxazepam-d5, lorazepam-d4, flunitrazepam-d7 and 7-aminoflunitrazepam-d7 were purchased from Promochem (Teddington, UK).

8.3.4 Preparation of Mobile Phase

A mobile phase consisting of 3 mM ammonium formate and 0.001% formic acid in water was prepared by adding 0.189 g ammonium formate and 10 μL of concentrated formic acid into a 500 mL volumetric flask and making up to 500 mL with deionised water.

8.3.5 Preparation of Solvents for the Synthesis of the Imprinted Polymers

8.3.5.1 Porogen - Ethanol-free chloroform

Ethanol-free chloroform was prepared by washing HPLC grade chloroform twice with an equal volume of water in a separating funnel. The chloroform layer was collected and dried over anhydrous calcium chloride. The chloroform was then filtered and collected in a round bottom flask. Further drying was then carried out over phosphorus pentoxide by refluxing the chloroform in a Soxhlet extractor at 70°C. Ethanol-free chloroform was collected and put in an amber glass bottle with activated 4 Å molecular sieve.

8.3.5.2 Cross-linker - EGDMA

EGDMA was washed with 10% aqueous NaOH in brine. It was then dried over anhydrous sodium sulphate and distilled under reduced pressure.

8.3.5.3 Monomer - MAA

MAA was dried over anhydrous sodium sulphate and distilled under reduced pressure.

8.3.5.4 Initiator - AIBN

AIBN was dissolved in methanol at room temperature,then the solution was filtered and kept in a freezer to recrystalize.

8.3.6 Synthesis of Flunitrazepam MIP

The MIP for flunitrazepam was prepared by dissolving flunitrazepam (0.2424 g, 0.77 mmol), MAA (0.3996 g, 4.64 mmol) and EGDMA (4.6004 g, 23.21 mmol) in ethanol-free chloroform (6.7 mL) in a 25 mL, thick-walled glass Kimax tube. AIBN (0.0838 g, 0.51 mmol) was then added and the solution sparged with oxygen-free nitrogen for 5 min while cooling in an ice bath. The sealed Kimax tube was thermostated at 4°C, to facilitate template-monomer complex formation, and irradiated with a Blak-Ray longwave UV lamp (model B-100A) for 24 hours. Thereafter, the polymer monolith obtained was transferred to a water bath set at 60°C for a further 24 hours, to complete the cure of the polymer. A non-imprinted polymer (NIP) was prepared in the same manner as the MIP but in the absence of template (flunitrazepam).

The MIP and NIP monoliths were crushed, mechanically ground and wet-sieved using acetone to deliver polymer particulates in the 25-38 μm size range. The template was extracted from the MIP by extensive washing with a mixture of methanol and acetic acid (9/1, v/v) for 24 hours. The polymer particles were then dried under vacuum at 60°C prior to use.

8.3.7 Preparation of MISPE

20 μm polyethylene frits were inserted into empty 1 mL polypropylene SPE cartridges. The cartridges were connected to a vacuum manifold. 20 mg of the MIP or NIP was

slurried with acetonitrile and packed into the cartridges. The cartridges were subjected to vacuum for 30 s before insertion of a second frit on top of the sorbent bed.

8.3.8 LC-MS-MS Analysis

LC was carried out using 3 mM ammonium formate and acetonitrile. The elution program consisted of a linear gradient (65-20%) of 3 mM ammonium formate for 13 minutes. The percentage of ammonium formate was decreased to 10 % between 13 to 13.5 minutes. It was then held at 10% between 13.5 to 16.5 minutes before being increased back to the initial conditions (65%) between 16.5 to 20 minutes. 20 µl samples were injected onto the Gemini C18 column at a flow rate of 0.3 mL min^{-1}. Ionization of analytes was performed using electrospray ionisation (ESI) in the positive ion mode. The nitrogen sheath and auxiliary gas flow rates and collision energy were set at the optimized conditions for each analyte.

All mass spectral data was collected in electrospray positive ion mode. The capillary temperature, sheath and auxiliary gas flow rates and collision energies were optimized for each analyte. The probe voltage used was 4.5 kV. Internal standard data was collected in selected ion monitoring (SIM) mode and analytes were identified on the basis of their retention time and full MS-MS spectra. The product ion ratios were monitored to gain further qualitative identification data. The optimum tuning parameters, precursor and product quantitation ions are shown in Table 6-2.

8.3.8.1 Preparation of Hair Samples

Prior to analysis, hair samples were decontaminated and pre-extracted using method described by Miller *et al.* [143]. Hair samples were washed with 0.1% aqueous sodium dodecyl sulfate (SDS) and sonicated for 10 minutes. The hair samples were then rinsed and sonicated for 10 minutes, twice with deionised water and twice with dichloromethane, then left to dry in air. Washed hair samples were then weighed out and approximately 30 mg samples transferred into vials for the hair pre-extraction process.

In the hair pre-extraction process, 1.5 mL of methanol:25% aqueous ammonium hydroxide (20:1, v/v) was added to each vial containing a hair sample, sonicated for one hour and left overnight at room temperature. The extraction solvent was removed and transferred from the vial to a test tube. The hair was then washed twice with 0.75 mL of

methanol: 25% aqueous ammonium hydroxide (20:1, v/v) and the washings removed and transferred to the same test tube as the first extract. The contents of the test tubes were evaporated to dryness under a stream of nitrogen gas and reconstituted with 1 mL of toluene prior to extraction with the diazepam MISPE.

8.3.8.2 Template removal

In order to totally remove the template, the MIP was treated in series with 1 mL acetonitrile, 1 mL chloroform and 1 mL of 30% acetic acid in acetonitrile. After each cycle of treatment, the 30% acetic acid/ acetonitrile fraction was collected, evaporated and reconstituted with LC-MS mobile phase prior to analysis. In between treatment series, the MIP sorbent was allowed to dry.

The results showed that, even after treatment ten times with washing solutions, the template is still retained within the imprinted polymer. (Figure 8-1). The same observations were reported by Andersson *et al.* [108]. Furthermore, 7-aminoflunitrazepam, a flunitrazepam metabolite was also detected in the eluent. This metabolite should not be found in the eluent since only pure flunitrazepam had been used during the synthesis. There is a possibility that 7-aminoflunitrazepam was produced during the polymerisation process. Another explanation might be a contamination of the flunitrazepam standard with 7-aminoflunitrazepam. A preparation of a standard solution of 100 ng flunitrazepam analysed using LC-MS-MS produced a chromatogram with a clear peak for 7-aminoflunitrazepam, thus confirming the contamination of the standard. Therefore, the performance of flunitrazepam MISPE was assessed only with other benzodiazepines *i.e.* diazepam, nordiazepam, oxazepam, temazepam, lorazepam, chlordiazepoxide and nitrazepam.

It was also observed that the concentration of flunitrazepam remaining in the MIP after extensive washing was about five times higher than that of diazepam in the anti-diazepam MIP, although the initial concentrations for both templates added in the polymer mixture were about the same. It was probably due to a stronger interaction between the flunitrazepam template and monomer during polymerization, making it more difficult to extract from the polymer network.

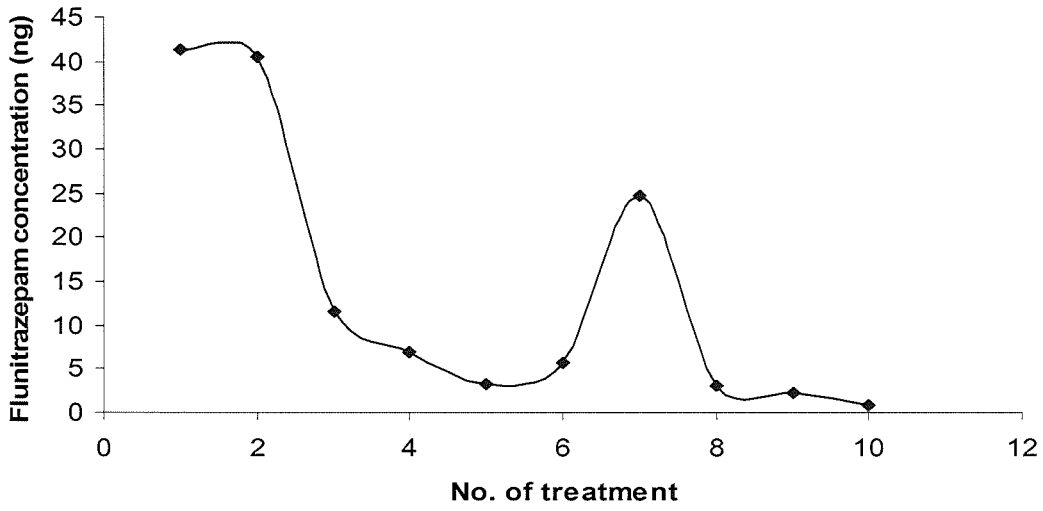


Figure 8-1 Concentration of flunitrazepam in the eluate after each treatment.

8.3.9 Validation of flunitrazepam MISPE for hair samples

8.3.9.1 Recoveries of benzodiazepine drugs

Benzodiazepine derivative drugs, namely oxazepam, lorazepam, chlordiazepoxide, temazepam, nordiazepam, nitrazepam and diazepam, were used to probe the group selectivity of the flunitrazepam MIP.

50 ng samples of each drug were prepared in 1 mL toluene. The cartridge was preconditioned with 0.5 mL of toluene. Each sample was passed through a cartridge and washed with 0.3 mL toluene. The analytes retained in the cartridge were eluted with 0.5 mL 15% (v/v) acetic acid in acetonitrile. 100 ng of internal standard was then added after the extraction.

8.3.9.2 Limits of Detection (LOD) and Limits of Quantification (LOQ)

To demonstrate the sensitivity of this method for forensic analysis of benzodiazepines in hair samples, an experiment was carried out to determine the LOD and LOQ values of the benzodiazepines. The LOD and LOQ were determined for each drug using spiked hair. 30 mg of blank, decontaminated hair was spiked with 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 2 and 5 ng of each drug and 100 ng of the internal standard mixture. The samples were then pre-extracted, extracted with MISPE and analyzed with LC-MS/MS.

8.4 Results and Discussion

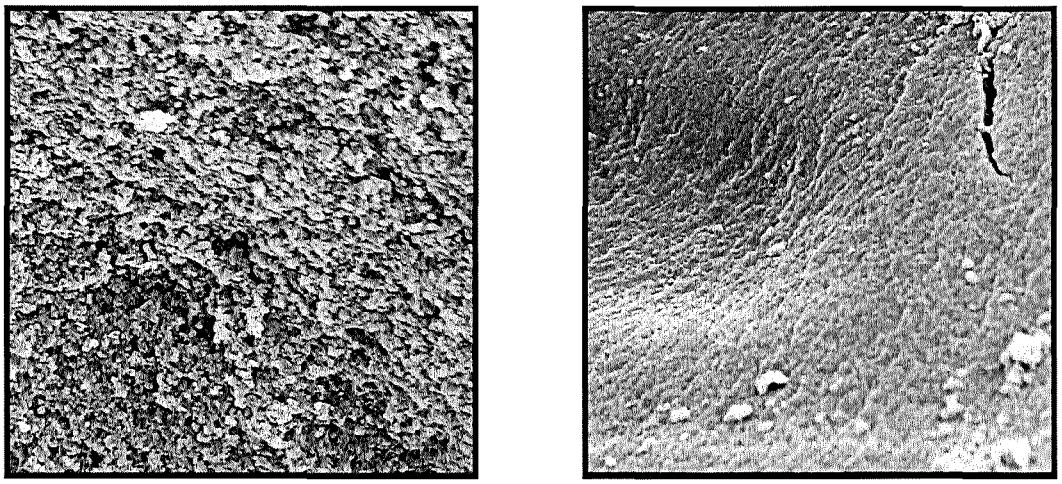
8.4.1 Recoveries

The percentage recoveries are summarized in Table 8-1. It was clear that the MIP exhibited good cross-reactivity, recognizing most benzodiazepine analogues, except for temazepam and chlordiazepoxide. The blank polymer showed lower recoveries for all benzodiazepines with unsatisfactory percentage RSD values.

Table 8-1 Percentage of recovery values for benzodiazepines using flunitrazepam MISPE

Analyte	Mean % Recovery (% RSD for n=5), Amount added = 50 ng/ 30mg hair	
	MIP	NIP
Oxazepam	87.2 (7.1)	62.8 (19.6)
Lorazepam	82.5 (6.7)	69.4 (10.5)
Chlordiazepoxide	74.7 (9.3)	65.1 (21.0)
Temazepam	42.6 (8.4)	36.1 (12.6)
Nordiazepam	87.5 (8.3)	73.2 (29.9)
Nitrazepam	82.5 (6.7)	77.0 (11.0)
Diazepam	86.5 (7.3)	51.2 (10.3)

The lower percentages recoveries with the NIP are probably due to poor recognition compared to the MIP. The findings show similar behaviour to diazepam NIP. The SEM micrographs in Figure 8-2 show low porosity of the surface of the NIP compared to the surface of the MIP.



(a) (b)

Figure 8-2 SEM micrograph of (a) flunitrazepam MIP and (b) NIP (x 10,000)

8.4.2 Limits of Detection (LOD) and Limits of Quantification (LOQ)

The correlation coefficients (r^2) of the calibration curves were greater than 0.99 for all analytes. The values are shown in Table 8-2.

Table 8-2 Calibration data for the benzodiazepines using flunitrazepam MIP

Analyte	r^2	b	a
Oxazepam	0.9950	0.0197	-0.0200
Lorazepam	0.9916	0.0268	0.0023
Chlordiazepoxide	0.9901	0.042	-0.0065
Temazepam	0.9960	0.0233	-0.0038
Nordiazepam	0.9924	0.0028	-0.0005
Nitrazepam	0.9909	0.0088	0.0011
Diazepam	0.9938	0.0008	-0.0017

Typical equation is $y = bx + a$, where x is the analyte concentration and y is the peak area ratio of the analyte and internal standard.

The LODs and LOQs obtained for benzodiazepines are shown in Table 8-3.

Table 8-3 The LOD and LOQ values for the benzodiazepines using flunitrazepam MIP

Analyte	LOD (ng/mg)	LOQ (ng/mg)
Oxazepam	0.01	0.02
Lorazepam	0.02	0.04
Chlordiazepoxide	0.02	0.04
Temazepam	0.01	0.02
Nordiazepam	0.02	0.03
Nitrazepam	0.02	0.03
Diazepam	0.02	0.03

The LOD and LOQ values for flunitrazepam MISPE were lower than for diazepam MISPE. This was probably due to the solvent used in the washing step. The purpose of this solvent was to extract the background interferences arising from the matrix. In the diazepam MISPE application, the solvent probably extracted small amounts of benzodiazepines. However in flunitrazepam MISPE, this solvent was not strong enough to disrupt the interaction of benzodiazepines in the MIP binding cavity. Therefore, lower levels of benzodiazepines were detected in flunitrazepam MISPE.

8.4.3 Comparison of the Performance of Flunitrazepam MISPE with Diazepam MISPE

The percentage recoveries of benzodiazepines using flunitrazepam and diazepam MIPs compared to the corresponding NIPs is summarized in Table 8-4.

The differences in the percentage recoveries for most of the benzodiazepines between flunitrazepam MIP and its corresponding NIP were considered to be small. This indicated a lower binding affinity of the benzodiazepines toward the imprinted polymer. The diazepam MIP showed better affinities, where the percentage of recoveries was more than 30% than the diazepam NIP for most of the benzodiazepines obtained.

Table 8-4 Percentage recoveries of benzodiazepines on polymers prepared with flunitrazepam and diazepam template

Analyte	Mean % Recovery Amount added = 50 ng/30 mg hair		Differences in % recovery between flunitrazepam MIP and NIP	Mean % Recovery Amount added = 50 ng/30 mg hair		Differences in % recovery between diazepam MIP and NIP
	Flunitrazepam MIP	Flunitrazepam NIP		Diazepam MIP	Diazepam NIP	
7-Aminoflunitrazepam	-	-	-	91.9	71.0	20.9
Oxazepam	87.2	62.8	24.4	73.4	41.4	32
Lorazepam	82.5	69.4	13.1	97.2	48.1	49.1
Chlordiazepoxide	74.7	65.1	9.6	61.6	32.0	29.6
Temazepam	42.6	36.1	6.5	89.6	54.3	35.3
Flunitrazepam	-	-	-	39.0	2.3	36.7
Nordiazepam	87.5	73.2	14.3	102.9	65.0	37.9
Nitrazepam	82.5	77.0	5.5	92.3	60.6	31.7
Diazepam	86.5	51.2	35.3	93.0	16.3	76.7

'-' = not determined

In non-covalent imprinting, the intermolecular interactions between analyte and NIP are due to the interaction of the analyte with free carboxylic acid groups (from MAA) on the polymer surface. In MISPE this is considered to be a secondary interaction. In optimized conditions, an appropriate solvent should be able to disrupt the secondary interaction and leave only the stronger primary interaction of the MIP polymer and the analyte. When the same solvent is applied to NIP, the intermolecular interactions between the analyte and carboxylic acid groups on its surface will be totally disrupted.

In this case, the washing solvent used in diazepam MISPE procedure again did not provide optimum recognition conditions in flunitrazepam MISPE. Therefore, the diazepam MIP showed higher binding characteristics compared to the flunitrazepam MIP.

8.5 Conclusion

The experimental results in this work showed that the MIP prepared using flunitrazepam as the template had good recognition properties for other benzodiazepines drugs. The MIP clearly displayed class selectivity for benzodiazepines. The performance of both flunitrazepam and diazepam MISPE indicated that the application of MIPs in extraction procedures is very useful for forensic analysis purposes, especially when dealing with unconventional samples such as hair. These types of samples need a highly selective extraction method due to background interferences from the matrices. The LOD and LOQ results also showed that the MISPE in conjunction with LC-MS-MS method is highly sensitive to detect low level of benzodiazepines in hair samples.

9 SYNTHESIS OF AN MIP FOR PARAQUAT RECOGNITION USING ANALOGUE TEMPLATES

9.1 Introduction

The method for the extraction of paraquat and other QA compounds and their detection by LC-MS was established in Chapters 3 and 4. Good recovery and LOD values were obtained for paraquat. The results were also comparable with other SPE methods reported previously. So far, SPE has become the most reliable extraction technique for the extraction of paraquat from either environmental or biological samples. Recently, the coupling of molecular recognition mechanisms using MIPs with separation procedures has been established. This combination offers better specificity, selectivity and high tolerance of extreme pH and temperature conditions.

Currently, there is a significant number of published reports on the use of MISPE for the extraction of pesticides other than paraquat, such as bentazone, triazine, organophosphates, chlorinated phenoxyacetic acid, phenylureas and sulfonylureas from water and soil samples [146, 153, 154, 155, 156, 157, 158, 159, 160].

Zhu *et al.* reported a MISPE method for organophosphates [146]. This method was found to be reliable and efficient for the analysis of polar organophosphates in real water and soil samples compared to the LLE and conventional SPE methods published previously.

MISPE methods for sulfonylureas using various monomers, crosslinkers and porogens have also been reported [151, 154, 155]. All these methods showed good specificity and selectivity to the template and other structurally related sulfonylureas under optimized MISPE conditions. MISPE procedures for chlorinated phenoxyacid pesticides also showed a superior clean-up with good recovery values compared with C18-reversed-phase SPE [161].

One difficulty in the preparation of anti-QA MIPs is that the polymerisation reaction is carried out in a relatively non-polar solvent (the porogen) which by its nature is not a good solvent for the QA compounds which must be present to act as the template. Current research in the field of MIPs involves the creation of synthetic routes in polar

media, including aqueous systems, which should greatly extend the range of applications of MIPs [86]. For the present study, an alternative approach was used involving structural analogues of paraquat which do not have a QA functional group. A number of these were considered, including alicyclic double ring structures with tertiary amines in each ring and dipyridines. These are further discussed below.

9.2 Aims

The aim of this study was to prepare MIPs using paraquat analogues which could be dissolved in the porogen solvent and subsequently to assess their value for MISPE of quaternary ammonium compounds from biological specimens.

9.3 Experimental

9.3.1 Instrumentations

LC-MS-MS analysis of QA compounds was carried out using a Thermo Finnigan LCQ Deca XP (Thermo Finnigan, San Jose CA, USA) equipped with a surveyor autosampler and MS pump system. Chromatographic separation was performed using an Atlantis® dC18 column, 100 mm x 2.1 mm ID, 5 µm (Waters, USA).

9.3.2 Chemicals and Materials

Paraquat dichloride, diquat dibromide and difenzoquat were obtained from Promochem (Middlesex, UK). Ethyl viologen and heptafluorobutyric acid were obtained from Aldrich (Milwaukee, WI, USA). Ammonium formate was purchased from Fluka (Buchs, Switzerland). HPLC grade acetonitrile, formic acid, toluene, chloroform and dichloromethane were purchased from BDH (Poole, UK).

4,4'-dipyridine, 2,2'-dipyridine, 2,4'-dipyridine and 2,2'-azobisisobutyronitrile (AIBN) for polymer synthesis was obtained from Acros Organics (Geel, Belgium). 2-(Trifluoromethyl) acrylic acid (TFMAA) and ethylene glycol dimethacylate (EGDMA) were purchased from Aldrich (Steinhem, Germany).

Empty 1mL polyethylene SPE cartridges and frits (20 μm mean pore size) were purchased from International Sorbent Technology Limited (Mid Glamorgan, UK).

9.3.3 Standard

Stock standard solutions (1 mg/mL) of QA herbicides was prepared by dissolving the dry chemical powder in methanol and were stored at 4°C in plastic bottles to avoid binding of QA herbicides to the surfaces of glassware. Stock solution is diluted to obtain a working standard solution of 1 μg/mL.

9.3.4 Solutions

9.3.4.1 Preparation of Mobile Phase

Mobile phase was prepared by adding 0.6306 g of ammonium formate and 0.975 mL of HFBA to 450 mL deionised water in 500 mL volumetric flask. The volume was then made up to the mark with deionised water.

9.3.4.2 Preparation of pH 6.0 Phosphate Buffer

1.70 g of sodium hydrogen phosphate and 12.14 g of sodium dihyrogen phosphate were weighed into a 1 L volumetric flask and the solution was made up to volume with deionised water.

9.3.5 Preparation of Solvents for the synthesis of the Imprinted Polymers

9.3.5.1 Porogen - Ethanol-free Chloroform

Ethanol-free chloroform was prepared by washing HPLC grade chloroform twice with an equal volume of water in a separating funnel. The chloroform layer was collected and dried over anhydrous calcium chloride. The chloroform was then filtered and collected in round bottom flask. Phosphorus pentoxide was added then refluxed using Soxhlet extraction at 70°C. Ethanol-free chloroform was collected and put in an amber glass bottle with activated 4 Å molecular sieve.

9.3.5.2 Crosslinker - Divinyl Benzene (80%)

DVB-80% was purified by passing through an alumina column. The solvent was collected in a glass bottle and kept in the refrigerator at 4°C.

9.3.5.3 Initiator - AIBN

AIBN was dissolved in methanol at room temperature,then the solution was filtered and kept in a freezer to recrystalize.

9.3.6 LC-MS-MS Analysis

LC was carried out using HFBA (15mM)-ammonium formate buffer (20mM) adjusted to pH 3.30 with formic acid as solvent A, and 100% methanol as solvent B. The elution program consisted of a linear gradient from 5%-90% of solvent B within 18 min. 20 µl of sample were injected into the Atlantis® dC18 column at 30°C, operated at a flow rate of 0.2 mL/min.

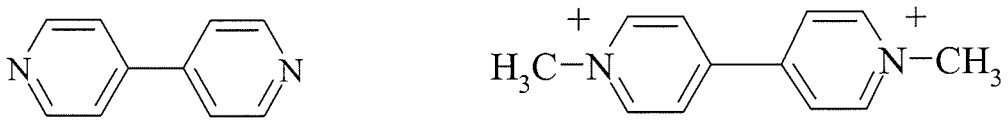
Ionization of analytes was performed using electrospray ionization (ESI) in the positive ion mode. A capillary temperature at 275°C was employed. The nitrogen sheath and auxiliary gas flow rates were set at 15 and 5 arbitrary LCQ units respectively. QA compounds were analysed with the MS parameters selected in Table 3-1 using product-ion scan MS-MS.

9.3.7 Synthesis of QA Herbicide MIP

9.3.7.1 Choice of analogue templates

In order to synthesize an MIP for paraquat, paraquat itself is not suitable as a template due to its physical and chemical properties. Paraquat is insoluble in most organic solvents; therefore the interaction between template and monomer in the porogen is unlikely to happen. In MIP preparation, a large number of complementary interactions between template and monomer are vital to increase the strength and fidelity of the recognition [84]. The stronger the interaction the higher the selectivity and specificity obtained. For this reason, analogues of paraquat were used which were soluble in the porogen solvent.

One of the analogues chosen was 4,4'-dipyridine. It is soluble in organic solvents and offers a similar molecular structure to paraquat, with both amine groups located at the same positions as the quaternary ammonium groups in the paraquat structure (Figure 9-1).



4,4'-dipyiridine paraquat

Figure 9-1 Molecular structure of 4,4'-dipyiridine and paraquat.

Furthermore, the tertiary amine groups should be able to interact with carboxylic groups from acrylic acid monomer to form a strong hydrogen bond. The possible interaction between 4,4'-dipyridine and monomer is illustrated in Figure 9-2.

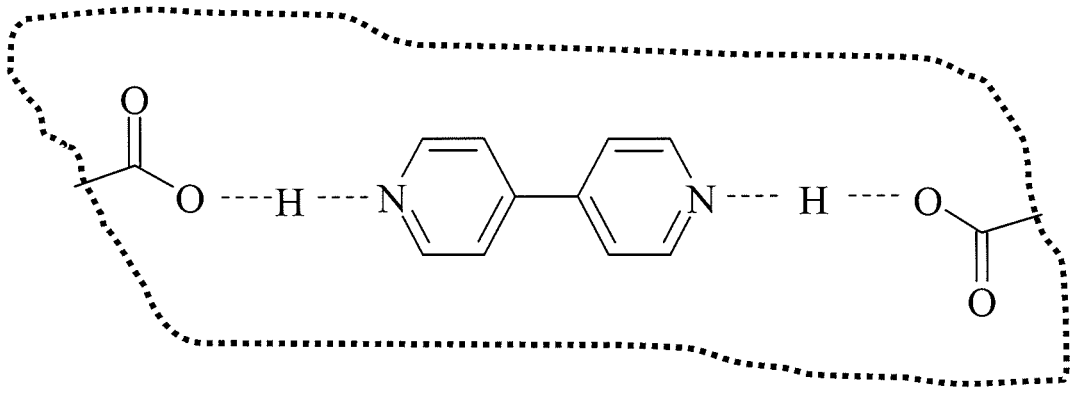


Figure 9-2 The interaction between 4,4'-dipyridine and MAA.

Another two template analogues, 2,2'-dipyridine and 2,4'-dipyridine were also selected to study the interaction of paraquat to binding sites created with similar dimensions and shapes of molecular structure but with different locations of the binding sites (Figure 9-3).

2,2'-dipyridine 2,4'-dipyridine

Figure 9-3 Molecular structure of 2,2'-dipyridine and 2,4'-dipyridine.

9.3.7.2 Choice of functional monomer and crosslinker

The common functional monomer used in non-covalent molecular imprinting is MAA. MAA has a carboxylic acid group which acts as a hydrogen bond donor and acceptor. In this study, in order to obtain a stronger interaction between template and monomer, TFMAA (Figure 9-4) was chosen as the monomer. TFMAA is more acidic than MAA and a better hydrogen donor due to the electron-withdrawing effect of the trifluoromethyl group.

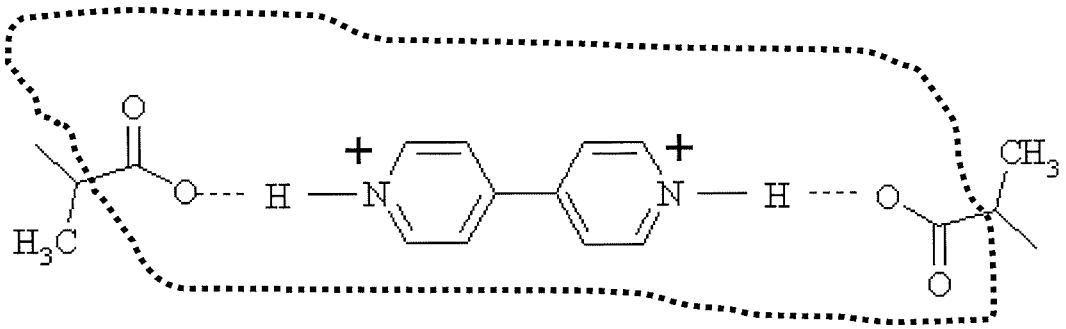


Figure 9-4 TFMAA interaction with 4,4'-dipyiridine

As for crosslinker, EGDMA would be a common choice. However, in a study reported by Yilmaz *et al.*, where TFMAA was used as the monomer, DVB offered lower non-specific binding compared to EGDMA [162]. Higher non-specific binding observed when TFMAA is combined with EGDMA resulted from the interaction of dissociable

acrylic acid compounds with the ester functionalities of EGDMA. In order to achieve as much selectivity as possible, and reduce non-selective binding, DVB was selected for use in this study.

Two MIPs for QA herbicides and one NIP were prepared in this experiment. The compositions of each MIP and of the NIP are shown in Table 9-1.

Table 9-1 The composition of MIP 1, MIP 2 and NIP

Polymer	4,4'-dipyridine g (mmol)	2,2'-dipyridine g (mmol)	2,4'-dipyridine g (mmol)	TFMAA g (mmol)	DVB g (mmol)
MIP 1	0.247 (1.58)	-	-	0.885 (6.31)	4.938 (37.93)
MIP 2		0.247 (1.58)	0.247 (1.58)	0.885 (6.31)	4.938 (37.93)
NIP	-	-	-	0.885 (6.31)	4.938 (37.93)

Each mixture was dissolved in 6.7 mL chloroform in a thick-walled glass Kimax tube. AIBN (0.0838 g, 0.51 mmol) was then added and the solution sparged with oxygen-free nitrogen for 5 min while cooling in an ice bath. The sealed Kimax tube was thermostated at 4°C, to facilitate template-monomer complex formation, and irradiated with a Blak-Ray longwave UV lamp (model B-100A) for 24 hours. Thereafter, the polymer monolith obtained was transferred to a water bath set at 60°C for a further 24 hours, to complete the cure of the polymer.

The resulting MIPs and NIP monolith were crushed, mechanically ground and wet-sieved using acetone to deliver polymer particulates in the 25-38 μm size range. The template was extracted from MIP 1 and MIP 2 by extensive washing with a mixture of methanol and acetic acid (9/1, v/v) for 24 hours. The polymer particles were then dried under vacuum at 60°C prior to use.

9.3.8 Preparation of MISPE Cartridges

20 μm polyethylene frits were inserted into empty 1 mL polypropylene SPE cartridges. The cartridges were connected to a vacuum manifold and 20 mg of the MIP 1, MIP 2 or NIP were slurried with acetonitrile and packed into the cartridges. The cartridges were subjected to vacuum for 30 s before insertion of a second frit on top of the sorbent bed.

9.3.9 MISPE Method Development

The study was aimed at evaluating the feasibility of using MISPE for the extraction of paraquat from blood samples. Therefore, a paraquat standard solution was prepared in water. The MISPE scheme employed a generic SPE protocol with the following general steps; i) conditioning the column with an appropriate solvent and sample condition, ii) loading the sample, iii) washing off possible interferences and iv) applying solvent to elute paraquat from the column.

9.3.9.1 Loading

MIPs and NIP were first conditioned with 1 mL methanol followed with 1 mL water. A standard solution of 100 ng paraquat was prepared in 1 mL deionised water and loaded in MIP 1, MIP 2 and NIP cartridges. The loading fractions were collected and analysed by LC-MS-MS.

The percentages of paraquat determined in the loading fractions from all cartridges were very low, showing that almost 100% of the analyte were retained in the column. This resulted from the non-specific binding of paraquat in the aqueous matrix due to the hydrophobic effect of the polymer [155, 163].

9.3.9.2 Elution and Washing

The effect of eluent solvents on paraquat binding to the polymers was studied to optimise the extraction condition. A standard solution of 100 ng paraquat was prepared in 1 mL pH 6.0 buffer solutions and loaded in MIP 1, MIP 2 and NIP cartridges. Next, the analyte was eluted with 0.5 mL of different types of solvent, listed in Table 9-2. The elution fraction was collected and analysed by LC-MS-MS.

Table 9-2 List of solvents used in elution step

Solvent	
A	1% Acetic acid in acetonitrile
B	2% Acetic acid in acetonitrile
C	5% Acetic acid in acetonitrile
D	1% Acetic acid in methanol
E	2% Acetic acid in methanol
F	5% Acetic acid in methanol
G	1% Trifluoroacetic acid in methanol
H	5% Trifluoroacetic acid in methanol

The percent recovery of paraquat in the eluent fraction was calculated using Equation 9-1 below. The recovery values for paraquat from MIP 1, MIP 2 and NIP using different solvents are showed in Figure 9-5. From the result, the highest percentage of paraquat eluted was obtained when using 0.5 mL of 1% trifluoroacetic acid in methanol as an elution solvent.

Equation 9-1

% Recovery = (peak area of elution fraction/peak area of 100 ng.mL^{-1} standard) x 100

To optimise the selectivity of the MIPs, the washing step was tried with chloroform, dichloromethane, acetonitrile, methanol, 1% of acetic acid in acetonitrile and 1% acetic acid in methanol. The washing fraction was collected and analysed using LC-MS-MS. The percentage recovery of paraquat collected from each cartridge is shown in Figure 9-6.

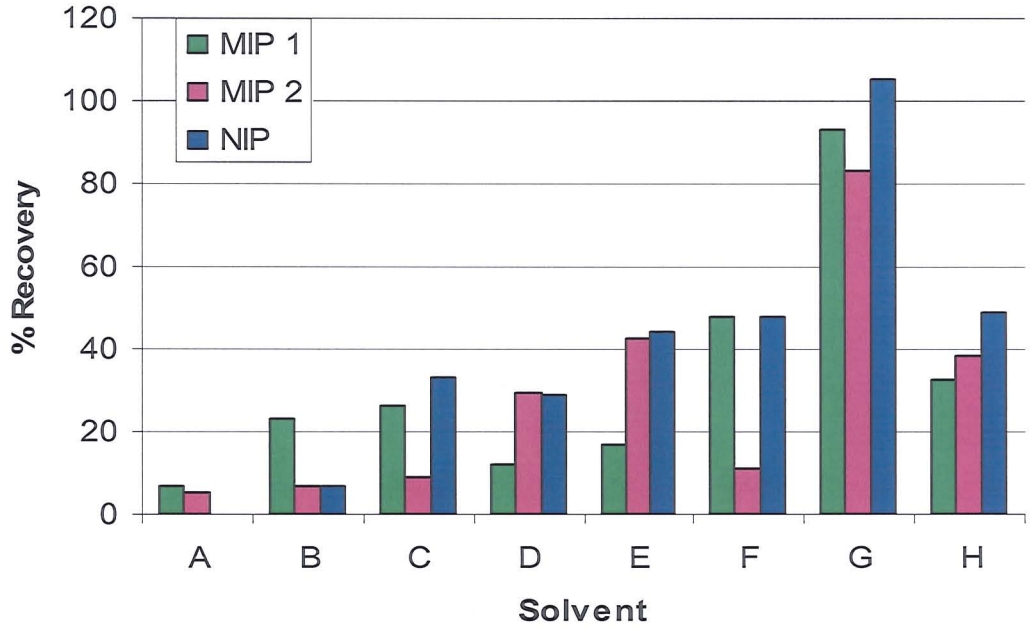


Figure 9-5 Percentage recovery in elution fraction collected using different solvents.

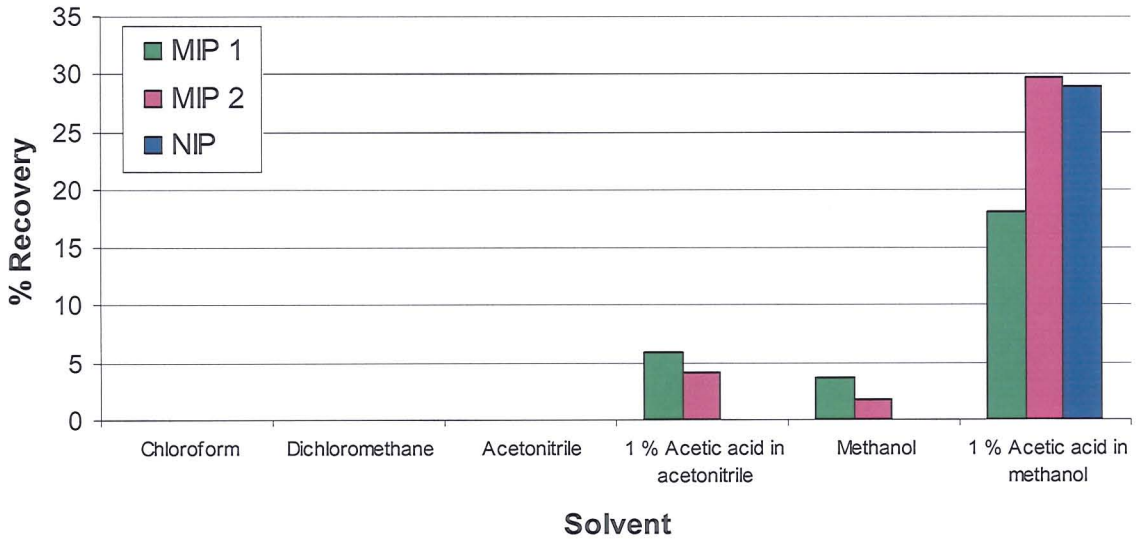


Figure 9-6 Percentage recovery in washing fraction collected using different solvents.

9.4 Discussion

The extraction profiles for paraquat were determined on MIP 1, MIP 2 and NIP. Following the loading of the samples from aqueous solution, there was a very small concentration of paraquat collected in the loading fraction in each cartridge, showing that the retention of paraquat was almost complete.

Elution of the paraquat was then undertaken using acetonitrile containing different percentages of acetic acid or methanol containing different percentages of either acetic acid or TFA. The recoveries from all cartridges were similar, with 1% TFA in methanol eluting the highest percentage of paraquat. Therefore, 1% TFA in methanol was used as an elution solvent.

In order to enhance the selectivity of the MIPs, the washing solvent was evaluated using more apolar solvents such as dichloromethane, toluene, acetonitrile, methanol, 1% of acetic acid in acetonitrile and 1% acetic acid in methanol. However, the effect was not significant and did not show any improvement in the selectivity of the MIPs.The results indicated an absence of any specific interactions of paraquat to the MIPs compared to the NIP. The retention of the analyte on the polymers was probably due to the interaction between analyte and free carboxylic acid group on the surface of the polymer. There is also a possibility that paraquat was unable to interact with the binding site due to its molecular size. The molecular size of paraquat is slightly bigger when compared to the templates. Furthermore, methyl groups on both sides of paraquat molecule may create steric hindrance and unable to fit into the binding site.

The morphology of the polymers was compared using SEM. Micrographs are depicted in Figure 9-7. The micrographs showed insignificant differences on the surface of the polymers.

The extraction and elution profiles represent the situation where only non-specific interactions were operating. Therefore, no further study was carried out.

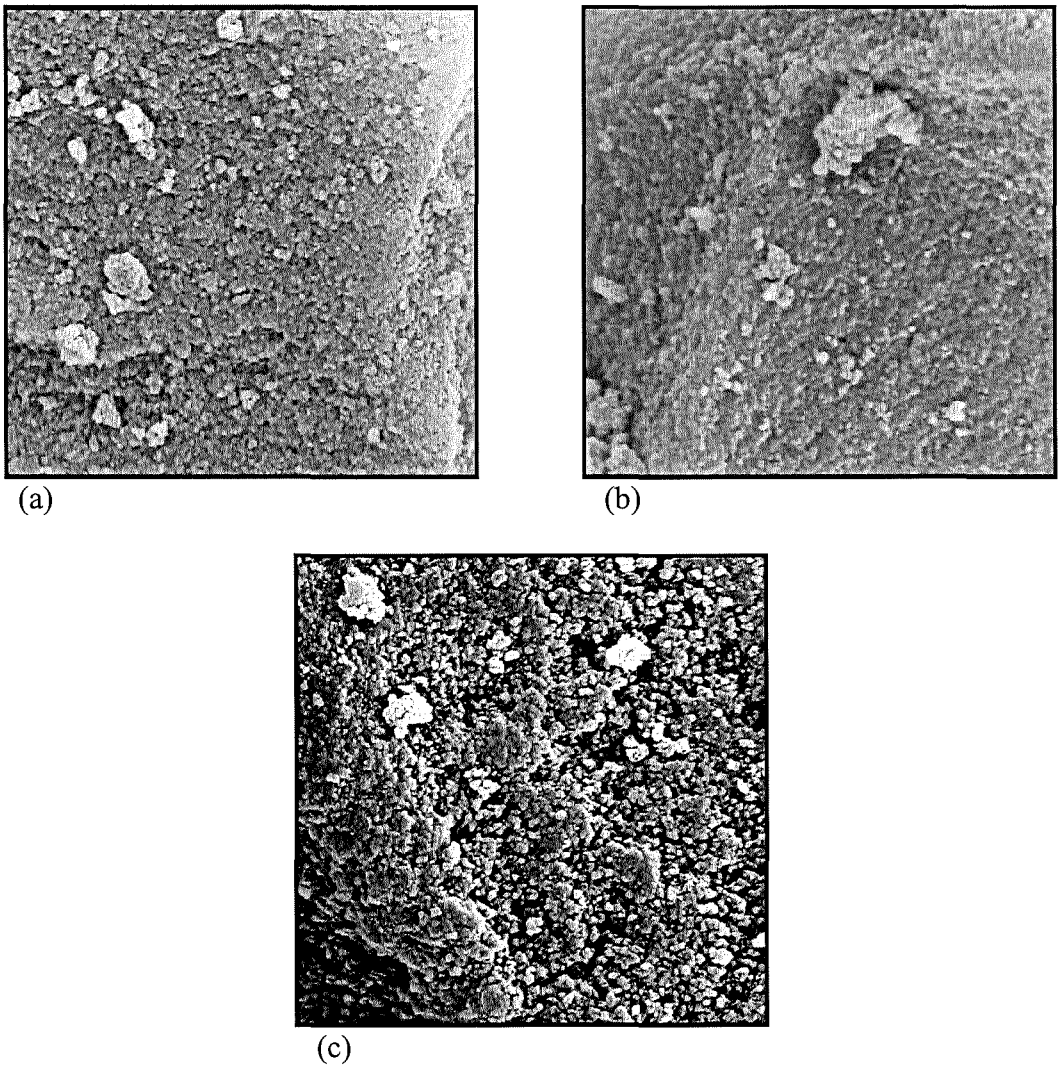


Figure 9-7 SEM micrograph of (a) paraquat MIP 1 and (b) Paraquat MIP 2 and (c) paraquat NIP (x 10,000)

9.5 Conclusion

Molecularly imprinted polymers for using 4,4'-dipyridine, 2,4'- dipyridine and 2,2'-dipyridine templates were prepared and applied for SPE for paraquat. The MIPs exhibited a lack of significant selectivity for paraquat compared to the NIP. The method failed to display a good recognition of the MIPs, a finding that might result from either poorly imprinted MIP or the incorrect choice of the polymer recipes. The non-specific interactions which occur with QA compounds would include ion exchange interactions, which are strong interactions that would perhaps dominate the overall solute-sorbent interaction. A non-acidic monomer, resulting in a neutral polymer, might allow the presence of molecular binding sites to be detected.

Therefore, it would be useful to study systematically the relation between the template species and functional monomer for better understanding of the molecular imprinting and recognition mechanism. Currently, computational molecular modelling has been studied in most molecular imprinting laboratories in order to predict the suitability of the proposed template molecule in terms of its compatibility towards the selected functional monomer and also to predict the possible interaction of the resultant polymer towards the analyte of interest.

10 GENERAL CONCLUSIONS AND FURTHER WORK

The detection of drugs of abuse and environmental or workplace pollutants in forensic cases has become a great challenge to toxicologists. Sensitive and reliable methods for the detection and determination of these poisons in biological samples are vital due to their widespread application and also due to the many poisoning deaths caused by them. The ideal analytical method must provide a highly selective extraction procedure that is able to isolate the analyte and allow detection without any interference or with minimal interference from the background matrix. In addition, a highly sensitive detection method is also desirable to gain a lower detection limit.

In the present work, a method based on SPE and LC-MS-MS was developed for the detection of quaternary ammonium compounds in human whole blood. This method provided an accurate assay for the simultaneous determination of quaternary ammonium herbicides and drugs. .Recoveries ranged from 79.7 to 105.1%, limit of detections were between 3.6 to 20.4 ng/mL and intra- and inter-day precisions were less than 18.6% at a concentration of 10 ng/mL. The method was successfully applied to paraquat poisoning cases. The developed method was very simple in practice, sensitive and reproducible and applicable to routine forensic analysis. The performance of the proposed method displayed better repeatability when compared to the previously reported methods by Lee *et al.*, with additional data on difenzoquat.[60, 164].

Studies on the application of molecular imprinted polymers as sorbents in SPE were also carried out. The first application was conducted using diazepam as a template molecule. The resulting MIP was used as the SPE sorbent. Results for diazepam analysis have been found to be accurate, reproducible and sensitive. The LOD and recovery values for diazepam by MISPE were 0.09 ng diazepam/mg hair and 93%, respectively, compared to 0.13 ng diazepam/mg hair and 69% using the SPE method described by Miller *et al.* [143]. The method also worked for other benzodiazepine drugs due to the group-selective binding nature of the MIP. The results arising from the application of this new method to post-mortem hair analysis were in excellent agreement with the ELISA method and corresponding blood sample results. The performance characteristics, in terms of sensitivity, selectivity and the cleanliness of the extracts, indicated that this method was sufficiently accurate and precise to be used for trace diazepam, diazepam metabolite and benzodiazepine analysis in hair samples, a

finding that may be of particular relevance to the investigation of drug-facilitated crime cases [165].

The extraction of selected benzodiazepines using diazepam MISPE followed by LC-MS-MS analysis provided cleaner extracts and better sensitivity when compared to a conventional SPE method. The MISPE method detected a higher number of diazepam positive case samples than the conventional SPE method because of the higher extraction recovery of the MISPE method due to the molecular recognition of the template molecule (diazepam) imparted into the sorbent by the imprinting process. The MISPE method has demonstrated selectivity for other benzodiazepine analogues and detected oxazepam in one sample where SPE detected none. The MISPE method was, however, not as selective for temazepam as the SPE method. Nordiazepam, oxazepam and temazepam concentrations, when detected using both extraction methods, were higher using the SPE method. Further work is required to investigate the ability of MISPE to detect benzodiazepines in single dose hair samples [166].

Similar results were obtained using flunitrazepam MISPE. The main problem arising from the MIP was due to bleeding of entrapped template. A vigorous and time consuming procedure was carried out to extract the template completely from the polymer. In the diazepam MIP, the template was successfully removed after washing with more than eight times with an appropriate extraction solvent. However, in the flunitrazepam MIP, this seemed to be unachievable.

In the later experiments directed towards the synthesis of a paraquat MIP, analogue templates were used. However, the resulting MIPs showed poor recognition of paraquat from an aqueous sample. This was probably due to inappropriate choice of analogues or maybe the poor recognition of the resulting polymers.

Currently, molecular imprinters prefer to predict the interaction between the selected templates and monomer or crosslinker using computer modelling prior to the actual experiment [167, 168, 169]. This approach is very important to gain a better understanding of the recognition process without wasting lots of processing time and materials. New methods of polymerisation have also been studied to obtain a more uniform shape and size of the polymer [110, 111, 170]. This type of polymer showed better performance than the polymer synthesised by bulk polymerisation in term of reproducibility and affinity.

The future application of MIP's in forensic toxicology samples will be very beneficial and definitely a worthwhile endeavour.

As stated earlier, the aim of this study was to investigate novel analytical methodologies for application to forensic toxicology problems. The work was successful but much more remains to be done in this area. Clearly, technology continues to advance and the LC-tandem MS method developed could be improved in terms of speed and sensitivity by using UPLC coupled to a triple quadrupole MS analyser. Applications of MIPs continue to be developed at an accelerating rate and the work done here has pointed to the potential value of these selective adsorbents in forensic toxicology. An MIP for benzodiazepines is of particular interest and merits further work because these molecules do not have any functional groups which readily lend themselves to ion exchange interactions at accessible pH values, and ion exchange is the primary basis for selective extraction of most other drug classes. Mixed success was achieved in this study with benzodiazepine MIPs and alternative reaction conditions, involving other monomers, cross-linkers and porogens should be investigated. Current research work on aqueous/polar reaction conditions would also be of interest for QA templates and other polar analytes, including peptides.

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APPENDIX 1: PUBLICATIONS IN SUPPORT OF THIS THESIS

1. Ariffin MM, Anderson RA. LC/MS/MS analysis of quaternary ammonium drugs and herbicides in whole blood. Proceedings of The International Association of Forensic Toxicologist 43rd Meeting, Seoul, Korea, 2005.

2. Ariffin MM, Miller EI, Cormack PAG, Anderson RA. Molecularly imprinted solid-phase extraction of diazepam and its metabolites from hair samples. Proceedings of The International Association of Forensic Toxicologist 44th Meeting, Ljubljiana, Slovenia, 2006.

3. Ariffin MM, Miller EI, Cormack PAG, Anderson RA. Benzodiazepines detection in hair by MISPE v. SPE. Proceedings of The International Association of Forensic Toxicologist 44th Meeting, Ljubljiana, Slovenia, 2006.

4. Ariffin MM, Miller EI, Cormack PAG, Anderson RA. Application of MISPE for benzodiazepine extraction from forensic hair samples. Proceedings of 4th International Workshop on Molecular Imprinting, Cardiff, UK, 2006.

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LC/MS/MS Analysis of Quaternary Ammonium Drugs and Herbicides in Whole Blood

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Abstract

Quaternary ammonium drugs (atracurium, bretylium, edrophonium, ipratropium, mivacurium, neostigmine, pancuronium and rocuronium) and herbicides (difenzoquat, diquat and paraquat) in human whole blood were analyzed by LC/MS/MS with positive ion electrospray ionization (ESI). The compounds were extracted with Bond Elut® LRC-CBA cartridges from whole blood samples containing internal standard. Internal standards for drug and herbicide analysis were benzyldimethylphenylammonium chloride monohydrate and ethyl viologen, respectively. The separations of quaternary ammonium compounds were carried out using ion-pair chromatography with heptafluorobutyric acid (15mM)-ammonium formate (20mM) buffer adjusted to pH 3.30 with formic acid and methanol gradient elution. The elution program consisted of a linear gradient from 5-90% of methanol within 18 minutes. The recoveries of all quaternary ammonium compounds ranged from 79.7-105.1%. The detection limits were between 3.6-20.4 ng/ml and the intra- and inter-day precisions were not greater than 18.6% for 10 ng/ml amount added.

The proposed methodology was applied to determination of paraquat in a case of accidental poisoning in which the victim is believed to have swallowed a quantity of paraquat which had been placed in a mineral water bottle. Both retention time and the product ion spectrum of paraquat in the sample matched well with those of the authentic standard. The concentration of paraquat in blood was 0.64 mg l^{-1} which is within the range associated with fatal paraquat poisoning.

Keywords: Quaternary ammonium compounds, Liquid chromatography-mass spectrometry

MOLECULARLY IMPRINTED POLYMER FOR SOLID-PHASE EXTRACTION OF DIAZEPAM AND ITS METABOLITES FROM HAIR SAMPLES

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Abstract

Background and Aim: Molecularly imprinted polymers (MIPs) are synthetic crosslinked polymers with strategically positioned binding sites for a selected analyte, creating the potential for MIPs to function as selective solid phase extraction (SPE) sorbents. An MIP is prepared *in-situ* by mixing the template, functional monomer and cross-linkers in a suitable porogen. After polymerization, the template is removed, leaving behind imprinted binding cavities within the polymer network. The aim of this study was to develop an anti-diazepam MIP to be used as a specific sorbent for SPE of benzodiazepines from hair.

Methods: An anti-diazepam MIP was synthesized based on work done by Vlatakis *et. al.* [G. Vlatakis, L. I. Andersson, R. Muller and K. Mosbach, *Nature*, 361 (1993) 645-647]. The resulting MIP was ground, sieved and packed in SPE cartridges. A molecularly imprinted solid-phase extraction (MISPE) procedure followed by LC-MS-MS analysis was optimised in the same manner as conventional SPE. The selectivity of the MIP sorbent was studied, using non-imprinted polymer as a control sorbent. The method demonstrated excellent accuracy and precision and good selectivity for diazepam. HPLC separation was carried out using a Gemini C18 column with gradient elution of 3 mM ammonium formate + 0.001% aqueous formic acid and acetonitrile at a flow rate of 0.3 ml min^{-1}. Ionization of analytes was performed using electrospray ionisation (ESI) in the positive ion mode. The nitrogen sheath and auxiliary gas flow rates and collision energy were set at the optimized conditions for each analyte.

Results: The recoveries of diazepam standards at concentrations of 20 and 50ng per 30 mg blank hair were 90.3% (RSD = 10.2%) and 93% (RSD = 1.5%), respectively compared to the non-imprinted polymer, which gave recoveries of 11.25 (RSD = 24.9%) and 16.3% (RSD = 17.1%) respectively. The LOD of diazepam in spiked hair samples was 0.09 ng per 30 mg hair. Also, extracts were cleaner than with conventional SPE, showing fewer interferences in LC-MS-MS. The method was also found to be applicable to the analysis of the diazepam metabolites nordiazepam, temazepam and oxazepam and other benzodiazepine drugs, including 7-aminoflunitrazepam, lorazepam, chlordiazepoxide, flunitrazepam and nitrazepam. The recoveries of 50 ng of standards of these drugs spiked into blank hair were between 39-102.9 % (RSD ≤ 17 %) and LODs ranged from 0.03 to 0.78 ng/mg. The MISPE procedure was applied

to the screening of post-mortem hair samples from drug related deaths and gave results which were in good agreement with those from enzyme linked immunosorbent assay (ELISA) data and also with the results of analysis of the corresponding autopsy blood samples using a validated in-house LC-MS method.

Conclusions: The performance characteristics of the method incorporating MISPE, in terms of sensitivity, selectivity, accuracy and precision indicate that it is applicable to the analysis of trace concentrations of diazepam and its metabolites in hair samples.

Keywords: Molecular imprinted polymer, benzodiazepines, hair samples analysis

Benzodiazepine Detection in Hair by MISPE v. SPE

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Abstract

Background: This preliminary study compares the results of benzodiazepine analysis in scalp hair samples from ten post-mortem cases using a validated conventional solid phase extraction (SPE) system and an anti-diazepam molecularly imprinted polymer solid phase extraction (MISPE) system.

Methods: The ten hair samples tested were from drug-related deaths in which a positive benzodiazepine blood result was obtained. The case samples were decontaminated with 0.1 % sodium dodecyl sulfate, distilled water and dichloromethane, incubated overnight in methanol/25 % ammonium hydroxide (20:1) and subsequently extracted by SPE or MISPE followed by LC-MS-MS analysis.

Results: Diazepam, nordiazepam, oxazepam, temazepam and nitrazepam were detected in the samples using both extraction methods. The recovery values for diazepam, nordiazepam, oxazepam, temazepam and nitrazepam in MISPE and SPE methods were between 73-103 % and 69-91 %, respectively. LOD levels between 0.06-0.39 ng/30mg hair and 0.03-0.24 ng/30mg hair were obtained with the MISPE and SPE methods, respectively. Diazepam was detected in more case samples using MISPE due to the lower LOD values and higher extraction recovery, as a result of excellent molecular recognition of the template. The LOD and recovery values for diazepam by MISPE are 0.09 ng/30mg hair and 93 %, respectively compared to 0.13 ng/30mg hair and 69 % by SPE. Nitrazepam levels detected by both methods were very similar. The MISPE method however was less sensitive for temazepam, nordiazepam and oxazepam determination compared to the conventional SPE method.

Conclusion: The molecularly imprinted polymer used for this study demonstrated good selectivity for the 5 benzodiazepines tested, especially the template substance diazepam. The MISPE method could be used as an alternative to conventional SPE extraction for the analysis of benzodiazepine positive hair samples collected from chronic users.

Keywords: Benzodiazepines; Hair; SPE; MISPE; LC-MS-MS

APPLICATION OF MISPE FOR BENZODIAZEPINES EXTRACTION FROM FORENSIC HAIR SAMPLES

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The aim of this study was to establish a MISPE protocol for benzodiazepines present in post-mortem hair samples. Benzodiazepines are the most frequently prescribed drugs in the world and are used as sedatives, hypnotics, muscle relaxants and anticonvulsants. As they are readily available, cheap and addictive, benzodiazepines are abused widely and sometimes taken simultaneously with alcohol and other drugs to amplify the drug-induced high. Determination of benzodiazepine drugs in hair samples is important when dealing with drug-facilitated crime cases, especially when crimes are reported 24 hours or more after consumption because commonly utilized samples such as urine and blood are no longer appropriate under such circumstances.

An anti-diazepam MIP was synthesized based on a synthetic protocol reported in the literature by Vlatakis *et al.*[1]. A MISPE procedure was optimized and analysis was followed by LC/MS/MS. The selectivity of the MIP sorbent towards nine benzodiazepines, namely diazepam, nordiazepam, oxazepam, lorazepam, chlordiazepoxide, temazepam, flunitrazepam, 7-aminoflunitrazepam and nitrazepam was studied, using a non-imprinted polymer (NIP) as a control sorbent. The method demonstrated excellent accuracy, precision and selectivity for diazepam and its metabolites nordiazepam, temazepam and oxazepam.

The MISPE protocol was validated and applied to ten post-mortem hair samples. The data obtained was compared to the data arising from a validated analysis method using a conventional SPE sorbent, which has a mixed mode cation exchange-hydrophobic phase extraction column. LOD levels between 0.03-0.78 ng/30mg hair and 0.03-0.62 ng/30mg hair were obtained with the MISPE and SPE methods, respectively. Diazepam, nordiazepam, oxazepam, temazepam and nitrazepam were detected in the post-mortem samples using both extraction methods. Diazepam was detected in more case samples using MISPE due to the lower LOD values and higher extraction recovery, as a result of excellent molecular recognition of the template. The LOD and recovery values for diazepam by MISPE are 0.09 ng/30mg hair and 93 %, respectively, compared to 0.13 ng/30mg hair and 69 % by SPE. Nitrazepam levels detected by both methods were very similar. The MISPE method was, however, marginally less sensitive for temazepam, nordiazepam and oxazepam determination compared to the conventional SPE method.

The performance characteristics of the method incorporating MISPE, in terms of sensitivity, selectivity, accuracy and precision, indicate that it is applicable to the analysis of trace concentrations of diazepam and its metabolites in hair samples. Furthermore, the MISPE extracts were cleaner than the extracts obtained by conventional SPE, leading to less interference in LC/MS/MS.

[1] G Vlatakis, LI Andersson, R Muller and K Mosbach, (1993), Drug assay using antibody mimics made by molecular imprinting, *Nature*, 361, 645-647.

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LC/MS/MS analysis of quaternary ammonium drugs and herbicides in whole blood☆

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Abstract

Quaternary ammonium drugs (atracurium, bretylium, edrophonium, ipratropium, mivacurium, neostigmine, pancuronium and rocuronium) and herbicides (difenzoquat, diquat and paraquat) in human whole blood were analysed by LC/MS/MS with positive electrospray ionisation (ESI), following extraction with Bond Elut® LRC-CBA cartridges. Internal standards were benzyldimethylphenylammonium chloride monohydrate and ethyl viologen for drug and herbicide analysis, respectively. Ion-pair chromatography used heptafluorobutyric acid (15 mM)–ammonium formate (20 mM) buffer adjusted to pH 3.30 with formic acid and a linear gradient from 5 to 90% methanol run over 18 min. Recoveries ranged from 79.7 to 105.1%, detection limits were between 3.6 and 20.4 ng/ml and the intra- and inter-day precisions were less than 18.6% at a concentration of 10 ng/ml. The method was applied to a case of accidental paraquat poisoning in which the concentration of paraquat in blood was 0.64 mg/l, which is within the range associated with fatal paraquat poisoning.

Keywords: Quaternary ammonium compounds; Liquid chromatography–mass spectrometry

1. Introduction

Quaternary ammonium (QA) compounds have been used as herbicides and anticholinergics drugs. Due to their toxicity, these compounds are found to be abused and encountered in many poisoning cases [1,2].

QA herbicides include two non-selective herbicides, namely paraquat and diquat and one highly selective herbicide, difenzoquat. For paraquat, an oral dose of only 1–2 g is fatal to most adults [3]. Paraquat is also believed to have deleterious effects on dopaminergic neurons and there is evidence suggesting that paraquat may be associated with the development of Parkinson's disease [4,5]. Diquat and difenzoquat poisoning are less common than paraquat poisoning, so that reports of human poisoning and animal experimental data for diquat and difenzoquat are less extensive than for paraquat.

QA drugs are normally used in adjunct to general anaesthesia to relax skeletal muscle. In the case of overdosing, it will cause prolonged muscle paralysis and changes in heart rate [6]. Some of these drugs have been known to be abused in equine sports [7].

Determination of QA compounds in various matrices by using gas-chromatography [8], liquid chromatography (LC) [9–11] and capillary electrophoresis [12] has been described. Ion-pairing liquid chromatography methods for determination of these compounds require additives to the mobile phase to improve the separation and resolution. Castro et al. [13] reported an LC/MS method for determination of QA herbicides by using volatile ion-pairing reagents; heptafluorobutyric acid (HFBA) has yielded satisfactory results.

In a forensic toxicology investigation, biological fluids such as whole blood, plasma or serum and urine are most commonly analysed. Screening of drugs in urine alone is not sufficient to support toxicological findings due to factors such as the amount of excretion and the time lapse after the intake of the drug. In cases like this, whole blood samples may be the sample of choice especially for deteriorated blood, when total separation of plasma and serum red cells is not possible [14]. In fatal cases,

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postmortem paraquat and diquat concentrations after 7 days of ingestion ranged from 0 to 0.44 mg/ml and 0–0.6 mg/ml, respectively [15]. In a suicidal case presented by Klys et al. [2], a post-mortem blood pancuronium level of 1.1 mg/ml was observed.

Reported data on the stability of QA drugs in post-mortem samples are limited. Farenc et al. reported that atracurium and its metabolite, laudanosine, were stable over 24 h in acidified plasma at room temperature [16]. Better stability of these compounds was displayed when stored at −30 °C. Pancuronium was found to be stable when stored at −20 °C in blood for up to seven months, however it degraded rapidly at 20 °C [17].

In the present work, we developed an efficient and simultaneous procedure for determining QA compounds in human whole blood by using weak cation exchange SPE and LC/MS/MS. The proposed method offers lower detection limits recovery and reproducibility when compared to previous methods [7,11] for most QA drugs in blood samples. For QA herbicides in blood analysis, this method displayed a slightly better repeatability when compared to the study done by Lee et al. [18], with additional data on difenzoquat.

2. Experimental

2.1. Materials

Paraquat dichloride, diquat dibromide and difenzoquat were obtained from Promochem (Middlesex, UK). Atracurium, bretylium besylate, edrophonium, ipratropium bromide and pancuronium were purchased from Sigma (St. Louis, MO, USA). Neostigmine bromide was from Roche (Hertfordshire, UK). Ethyl viologen and heptafluorobutyric acid were obtained from Aldrich (Milwaukee, WI, USA). Mivacurium chloride (Mivacron) was from Glaxo-Wellcome (Middlesex, UK). Rocuronium bromide (Esmeron) was from Ornagon (Oss, Holland). Benzyldimethylphenylammonium chloride monohydrate was obtained from Acros Organic (Geel, Belgium). Ammonium acetate and ammonium formate were purchased from Fluka (Buchs, Switzerland). Formic acid and methanol were of HPLC grade. Bond Elut® LRC-CBA cartridges were purchased from Varian (CA, USA).

2.2. Instrumentation

LC/MS/MS analyses of QA compounds were carried out using Thermo Finnigan LCQ Deca XP (Thermo Finnigan, San Jose CA, USA) equipped with a Surveyor HPLC system. Chromatographic separation was performed using an Atlantis® dC18 column 100 mm × 2.1 mm ID, particle size 5 μm (Waters, Milford, MA, USA).

2.3. Extraction procedure

Stock standard solutions (1 μg/ml) of QA compounds except for rocuronium bromide and mivacurium chloride were prepared by dissolving the dry chemical powder in methanol and stored at 4 °C in plastic bottles. Rocuronium bromide and mivacurium chloride were prepared by diluting the formulations to 1 μg/ml with methanol.

SPE with a weak cation exchanger (Bond Elut® LRC-CBA) has been used to extract QA compounds. The p*K*a of this cation exchanger is 4.8, thus adjustment of the eluent pH to below 2.8 facilitates the recovery of quaternary amines.

For QA herbicides, blood samples were prepared by diluting 1 ml of whole blood with 4 ml phosphate buffer (pH 6.0). Then the blood samples were vortex mixed and centrifuged for at 1121 × *g* for 10 min. Extraction cartridges were conditioned with 3 ml methanol followed by 3 ml phosphate buffer (pH 6.0). Each sample was loaded through a cartridge. It was then washed with 3 ml of phosphate buffer (pH 6.0) followed by 3 ml of methanol. The cartridge was eluted with 1 ml of 1.0 M HCl/methanol (70:30, v/v). The eluate was evaporated to dryness under nitrogen at 35 °C and the residue was dissolved in 1 ml of the initial HPLC mobile phase. The content was transferred to an autosampler vial for LC/MS/MS analysis.

Extractions for QA drugs were prepared in the same manner except for using ammonium acetate solution (pH 8.0) as a buffer. Except for atracurium, in the wash process, the cartridge was washed with 2 times of 3 ml buffer and no methanol was used since it will elute atracurium.

2.4. LC/MS/MS analysis

LC was carried out using HFBA (15 mM)–ammonium formate buffer (20 mM) adjusted to pH 3.30 by formic acid as solvent A, and 100% methanol as solvent B. The elution program consisted of a linear gradient from 5 to 90% of solvent B within 18 min. Twenty microlitres of samples were injected onto the Atlantis® dC18 column at 30 °C, operated at a flow rate of 0.2 ml/min.

Ionisation of analytes was performed using electrospray ionization (ESI) in the positive mode. A capillary temperature of 275 °C was employed. The nitrogen sheath and auxiliary gas flow rates were set at 15 and 5 arbitrary LCQ units, respectively. QA compounds were analysed with MS parameters selected in Table 1 using product-ion scan MS/MS. Chromatograms of quantitation ions for all QA compounds are shown in Fig. 1.

2.5. Matrix effect study

This study was conducted to assess the interference caused by blood matrix during extraction. Five replicates of 1 ml blank blood were spiked with 50 ng of QA compounds and another five of standard line were prepared by spiking with the same concentration of QA compounds in 4 ml of loading buffer. All of the samples were vortexed, centrifuged and extracted using the described SPE procedure. One hundred nanograms of internal standard was added after the extraction. The percentage of matrix effect was calculated according to Eq. (1), where *a* and *b* are the peak area ratios of the analyte to internal standard in neat solution and blood extract, respectively.

$$\text{Percentage of matrix effect} = \frac{b}{a} \times 100\% \quad (1)$$

Table 1
MS parameters for the QA compounds by using LC–MS–MS in the positive ESI mode

QA compound	Precursor ion (m/z)	Product ions (m/z)	Relative Intensity	Collision energy (eV)
Atracurium	358	206.1	100.0	30
		207.1	8.40	
		327	5.30	
Bretylium	242	169.0	100.0	32
		71.9	20.2	
		224.9	17.8	
Diquat	183	157.1	100.0	39
		164.8	47.8	
		149.8	10.3	
Difenzoquat	249	208.1	100.0	47
		193.2	87.5	
		131.2	38.34	
Edrophonium	166	138.0	100.0	34
		137.0	32.3	
		148.8	12.7	
Ipratropium	332	166.1	100.0	35
		290.1	8.9	
		167.7	7.6	
Mivacurium	514	357.1	100.0	33
		428.2	68.3	
		342.3	44.5	
Neostigmine	223	208.0	100.0	40
		177.0	63.9	
		209.0	118	
Pancuronium	286	236.6	100.0	24
		100.0	9.3	
		206.8	12.3	
Paraquat	185	171.1	100.0	35
		166.8	52.3	
		166.0	12.3	
Rocuronium	529	487.2	100.0	32
		488.2	25.5	
		358.2	3.55	
Benzyldimethylphenyl-ammonium (Internal standard for drugs)	212	120.0	100.0	30
		121.1	40.2	
		134.0	37.4	
Ethyl Viologen (Internal standard for herbicides)	213	185.1	100.0	37
		195.0	86.5	
		194.2	3.2	

A value of <100% indicates analyte ion supression whereas >100% indicates analyte ion enrichment due to the matrix interference.

2.6. Recovery study

QA compounds each at 50 and 100 ng/ml were spiked in 1 ml of a drug free human blood sample ($n = 6$) were processed using the SPE procedure. 100 ng of internal standard (ethyl viologen for herbicides and benzyldimethylphenylammonium chloride monohydrate for drugs) was then added after the SPE. The recovery was determined by comparing peak area ratios obtained from QA ions of the compounds to that of the internal standard versus the peak area ratios of the same concentration of pure standards and internal standard spiked in extracted blood blank matrix.

2.7. Linearity and determination of the LOD and LOQ

The regression equations for QA compounds extracted from human whole blood were subjected to linear regression analysis of peak area ratios to internal standard (100 ng) against the spiked analytes (5, 10, 25, 50, 100 and 200 ng/ml). The LODs were calculated using Eqs. (2) and (3), where Y_B is the intercept, S_B is the standard error of the regression line and m is the gradient [19].

$$Y_{LOD} = Y_B + 3S_B \quad (2)$$

$$LOD = \frac{Y_{LOD} - Y_B}{m} \quad (3)$$

LOQ was calculated using the same method except that 10 times the standard error of the regression line was used (Eqs. (4) and (5)).

$$Y_{LOQ} = Y_B + 10S_B \quad (4)$$

$$LOQ = \frac{Y_{LOQ} - Y_B}{m} \quad (5)$$

2.8. Intra- and inter-day precisions

The intra- and inter-day precisions were determined by analysing 10, 50 and 100 ng/ml extracted spiked analytes in human whole blood six times in the same day and repeating this process on 6 separate days.

3. Results and discussion

3.1. Optimisation of the mobile phase

Initially, a mobile phase consisting of 15 mM HFBA (solvent A) and 100% methanol (solvent B) were used to separate QA compounds. HFBA was used as ion-pairing reagent to improve the separation and retention of QA herbicides [18]. Methanol was recommended over acetonitrile as an organic modifier to avoid a white film deposit on the ESI source [13].

However, when using this mobile phase, peaks obtained from paraquat and diquat separations were very poor and the detections were less sensitive. Therefore, experiments were carried out by adding 20 mM ammonium formate or 20 mM ammonium acetate to solvent A and adjusted to pH 3.30 with formic acid. Ammonium acetate and formic acid were recommended to improve the ionisation in the MS [20]. However, the ammonium formate mobile phase was found to be more sensitive and therefore was chosen for the determination and confirmation of QA compounds in LC/MS/MS.

3.2. Matrix effect

From the matrix effect results (Table 2), blood matrix components are found to cause interference with solid-phase extraction of the analytes especially for edrophonium and paraquat. Therefore, all standard solutions were prepared in whole blood matrix to mimic the actual sample conditions.

3.3. Quality parameters

The regression analysis between peak area ratios of QA compounds over the internal standard showed a good linearity in the

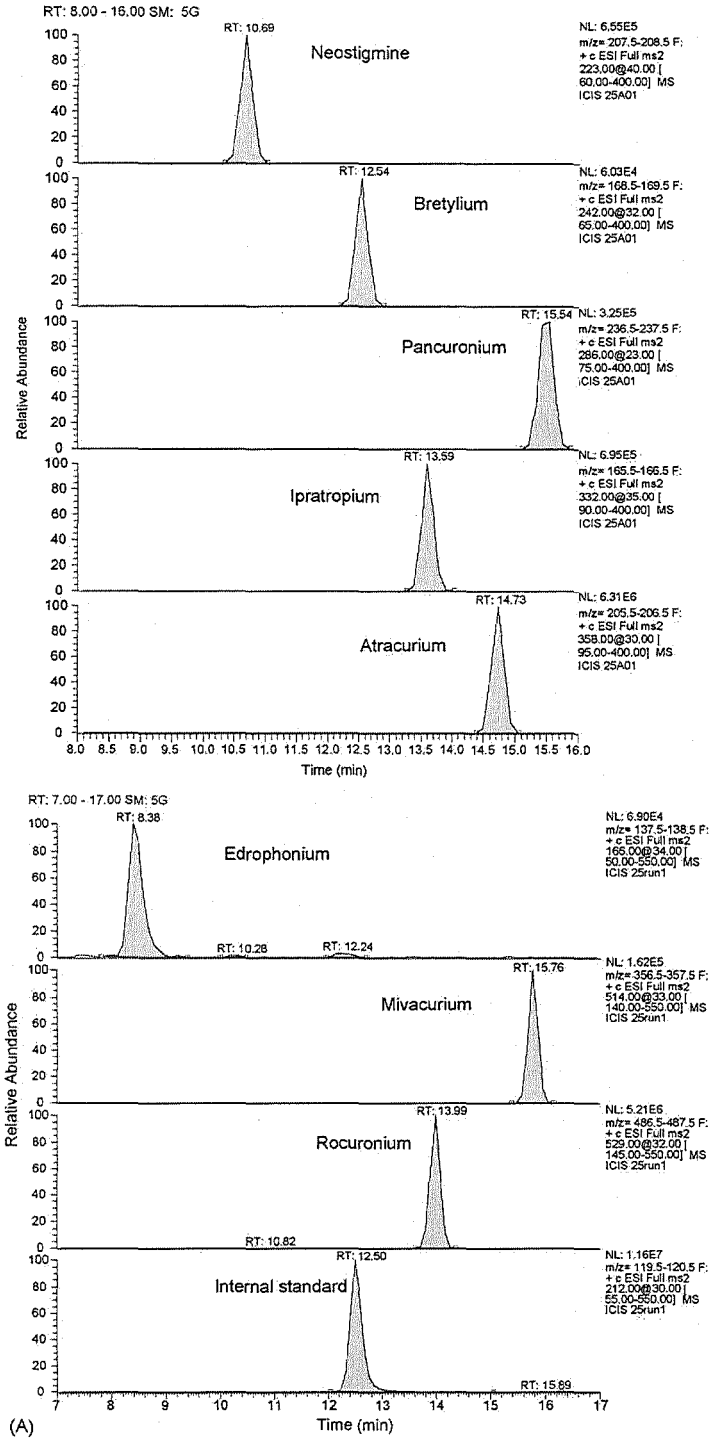

Fig. 1. (A) Chromatograms of the quantitation ions of QA drugs in blank blood spiked at a concentration of 25 ng/ml. (B) Chromatograms of the quantitation ions of QA herbicides in blank blood spiked at a concentration of 50 ng/ml.

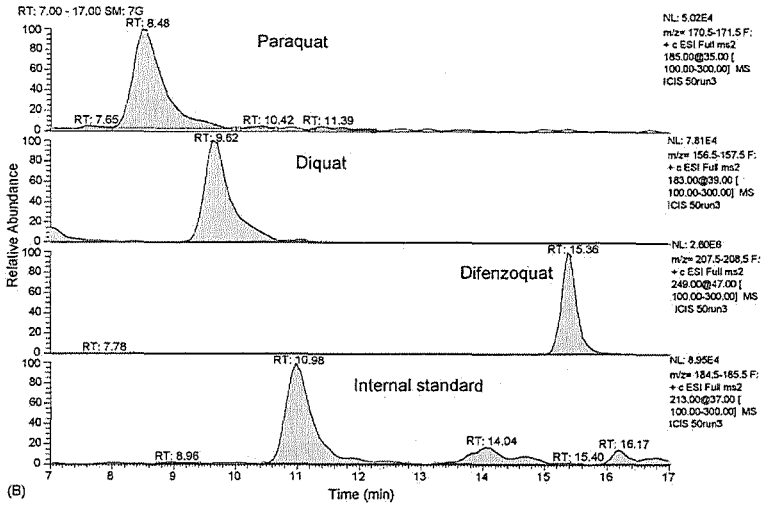

Fig. 1. (*Continued*).

Table 2
Blood matrix effect on sample extraction

QA compound	% Matrix effect
Atracurium	105.9
Bretylium	91.1
Diquat	101.2
Difenzoquat	84.2
Edrophonium	65.0
Ipratropium	78.8
Mivacurium	105.9
Neostigmine	85.5
Pancuronium	112.7
Paraquat	144.7
Rocuronium	86.6

range of 5–200 ng/ml. The correlation coefficients (r) for calibration curves were greater than 0.99. LODs and LOQs for all QA compounds are shown in Table 3.

Recoveries of QA compounds in human whole blood samples are presented in Table 4. The recoveries of all quaternary ammonium compounds ranged from 79.7 to 105.1%. The average recoveries and 100% value (null hypothesis) were not significantly different according to the calculated t-values, which were lower than the tabulated t-value of 2.5706 for $P = 0.05$.

Intra- and inter-day precisions results show that good precision can be obtained with the method described above. Relative standard deviations % (R.S.D.s) from intra- and inter-day precision ranging from 0.7 to 18.6% are shown in Table 5. At the LOQ level, 20% for precision and accuracy is acceptable [21].

4. Application in real sample

A 66-year-old man died 4 days after suffering from poisoning despite active therapy. A few days before he died, the deceased accidentally brought home three mineral water bottles containing Dextreone weedkiller from his workplace and placed them in his grocery bag. A friend, who helped him with his groceries, put the bottles in the fridge, believing them to be blackcurrant juice. Post mortem examination revealed jaundice, bilateral pleural

Table 3
Calibration, LOD and LOQ for QA compounds in human whole blood obtained using the proposed method

QA compound	r^2	b	a	LOD (ng/ml)	LOQ (ng/ml)
Atracurium	0.9994	0.0385	−0.0691	5.8	19.3
Bretylium	0.9997	0.0020	−0.0004	4.1	13.7
Diquat	0.9985	0.0038	0.0092	5.0	16.7
Difenzoquat	0.9997	0.0186	−0.0012	4.1	13.7
Edrophonium	0.9989	0.0003	0.0006	10.8	35.9
Ipratropium	0.9994	0.0115	−0.0148	5.9	19.5
Mivacurium	0.9927	0.0008	−0.0063	20.4	68.0
Neostigmine	0.9998	0.0075	−0.0059	3.6	12.1
Pancuronium	0.9981	0.0079	−0.0325	10.4	34.5
Paraquat	0.9984	0.0046	−0.0140	11.1	36.9
Rocuronium	0.9996	0.0180	−0.0264	4.9	16.2

Typical equation is $y = bx + a$, where x is the analyte concentration and y is the peak area ratio of analyte and internal standard. The calibration curves were determined over the range 5–200 ng/ml.

Table 4
Recoveries for QA compounds in human whole blood obtained from the proposed method

QA compound	50 ng/ml		100 ng/ml	
	Mean recovery (%)	R.S.D. of recovery (%)	Mean recovery (%)	R.S.D. of recovery (%)
Atracurium	105.1	3.8	94.3	3.1
Bretylium	79.7	17.5	90.7	15.7
Diquat	99.7	10.5	98.3	2.5
Difenzoquat	99.9	9.4	100.4	5.9
Edrophonium	95.2	3.1	90.2	6.6
Ipratropium	103.1	4.9	96.1	6.5
Mivacurium	93.1	8.1	92	3.8
Neostigmine	101.5	5.8	90.6	4.4
Pancuronium	94.3	7.6	96.5	2.1
Paraquat	84.9	2.8	89.7	5.7
Rocuronium	102.4	4.7	97.2	6.6

effusions, marked pulmonary oedema and mottling of the kidney indicative of death as the result of multiorgan failure. In addition there was an ulcerative oesophagitis and microscopic examination of the lungs revealed changes in keeping with ingestion of paraquat and its toxic effects. A blood sample was taken and the proposed methodology was applied to screening and confirmation of the paraquat level.

Quantification of paraquat was performed using a duplicate set of calibrators containing paraquat each at levels of 0, 10, 25, 50, 100, 200 and 300 ng/ml containing 200 ng/ml ethyl viologen as an internal standard. Fig. 2 shows the extracted ion chromatogram and product ion spectrum of paraquat standard. Both the retention time and the product ion spectrum of paraquat in the sample matched well with those of the authentic standard. This positive identification reveals the concentration of paraquat at 0.64 mg/l.

Table 5
Intra- and inter-day precision for QA compounds in human whole blood samples obtained using the proposed method

QA compound	Amount added (ng/ml)	Intra-day		Inter-day	
		Amount detected (ng/ml)	R.S.D. (%)	Amount detected (ng/ml)	R.S.D. (%)
Atracurium	10	10.4 ± 1.6	15.4	8.4 ± 1.5	17.5
	50	49.2 ± 3.6	7.2	47.6 ± 4.1	8.5
	100	96.3 ± 10.9	11.3	96.3 ± 7.2	7.5
Bretylium	10	10.4 ± 1.4	13.2	9.7 ± 1.3	13.8
	50	50.0 ± 4.6	9.1	47.7 ± 4.1	4.7
	100	100.0 ± 8.5	8.5	98.9 ± 6.0	6.1
Diquat	10	8.7 ± 1.6	17.9	10.7 ± 0.9	11.9
	50	46.0 ± 8.2	17.8	47.0 ± 0.3	0.7
	100	91.5 ± 5.5	6.0	96.0 ± 1.5	1.6
Difenzoquat	10	7.6 ± 1.1	14.3	10.7 ± 1.8	7.8
	50	47.1 ± 1.84	3.9	50.9 ± 2.4	4.8
	100	98.6 ± 3.1	3.1	102.2 ± 3.2	3.1
Edrophonium	10	10.1 ± 0.9	8.8	9.5 ± 1.4	14.2
	50	44.4 ± 4.8	10.7	51.1 ± 2.2	4.7
	100	94.2 ± 4.3	4.6	98.1 ± 5.3	5.4
Ipratropium	10	8.9 ± 0.7	8.2	9.4 ± 1.8	18.6
	50	47.7 ± 3.4	7.1	41.6 ± 2.9	6.9
	100	98.9 ± 7.1	7.2	98.2 ± 7.3	7.5
Mivacurium	10	10.1 ± 1.9	18.6	9.3 ± 1.7	18.4
	50	49.1 ± 4.4	9.0	48.6 ± 6.9	14.1
	100	101.8 ± 13.0	12.7	91.8 ± 11.7	12.8
Neostigmine	10	10.2 ± 1.3	12.8	9.7 ± 0.5	5.0
	50	49.9 ± 3.2	6.4	48.8 ± 2.8	5.7
	100	95.6 ± 5.6	5.9	96.6 ± 3.5	3.6
Pancuronium	10	8.5 ± 0.6	6.7	9.0 ± 1.3	14.3
	50	47.3 ± 0.94	2.0	49.0 ± 3.0	6.1
	100	94.5 ± 2.6	2.8	95.7 ± 7.5	7.8
Paraquat	10	10.1 ± 1.0	9.4	9.8 ± 1.5	15.2
	50	48.5 ± 4.8	9.8	49.9 ± 2.3	4.6
	100	103.6 ± 9.1	8.7	102.6 ± 3.3	3.2
Rocuronium	10	9.8 ± 1.3	13.4	10.0 ± .5	4.8
	50	49.7 ± 2.2	4.4	41.5 ± 2.8	6.7
	100	99.1 ± 3.5	3.6	99.5 ± 7.4	7.4

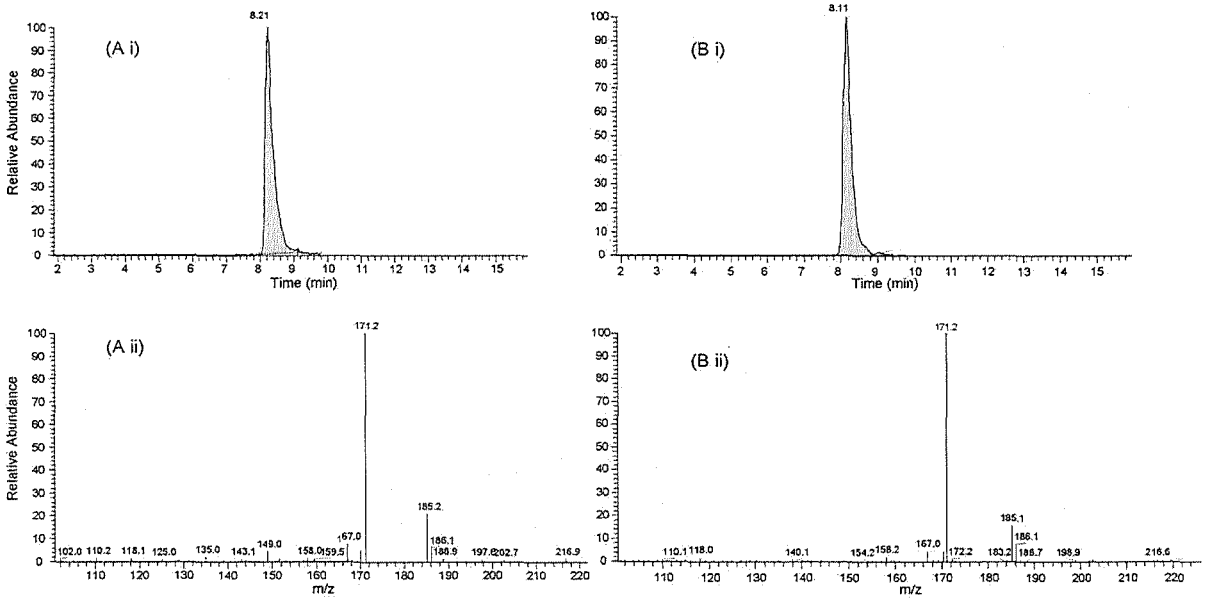

Fig. 2. (A) (i) extracted ion chromatogram of *m/z* 171 and (ii) product ion scan of paraquat *m/z* 185 $[M-H]^+$ for the poisoning sample; (B) (i) ion chromatogram of *m/z* 171 and (ii) product ion scan of paraquat *m/z* 185 $[M-H]^+$ of 300 ng/ml paraquat standard.

5. Conclusion

The proposed solid-phase extraction procedure and LC/MS/MS method provided an accurate assay for the determination of quaternary ammonium drugs and herbicides in human whole blood. Recovery and precision studies successfully quantified QA compounds in spiked human whole blood. The procedure has also been successfully applied to a fatal death case involving paraquat poisoning.

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Molecularly Imprinted Solid-Phase Extraction of Diazepam and Its Metabolites from Hair Samples

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An anti-diazepam, molecularly imprinted polymer (MIP) has been synthesized and used to extract diazepam and other benzodiazepines from hair samples via a molecularly imprinted solid-phase extraction (MISPE) protocol. Optimum retention of diazepam on the MIP columns was achieved using an apolar solvent, and the binding capacity of the polymer toward diazepam was found to be 110 ng of diazepam/mg of polymer. The recovery of a 50 ng diazepam standard spiked into blank hair was 93%, with good precision (RSD = 1.5%). The LOD and LOQ of diazepam in spiked hair samples were 0.09 and 0.14 ng/mg, respectively. The MISPE method was demonstrated to be applicable to the analysis of diazepam metabolites and other benzodiazepine drugs, in addition to diazepam itself. The application of the extraction method to postmortem hair samples yielded results that were in good agreement with the corresponding ELISA data (from blood samples) and data arising from the analysis of the same blood samples using a validated in-house SPE-LC–MS–MS method.

The technique of molecular imprinting generates synthetic materials that mimic the action of antibodies and enzymes. The production of molecularly imprinted polymers (MIPs) involves the synthesis of highly cross-linked polymers in the presence of template molecules. Post-synthesis, the template is removed, leaving behind imprinted binding sites (cavities) within the polymer network that are complementary in size, shape, and chemical functionality to the template. These binding sites are able to rebind the template molecule, or other molecules that have close structural similarity to the template molecule, in a strong and selective manner. The advantages that MIPs hold over natural receptors, such as their stability at extremes of pH and temperature, their ease of preparation, low cost, and reusability, have led to the development of various MIP applications, including chromatography,[1] catalysis,[2] chemical sensors,[3,4] and solid-phase extraction (SPE).[5,6] MIPs have been applied to the extraction of pollutants in the environmental area, such as river[7,8] and soil[9,10] samples. For biological samples, MIPs for drugs and toxins in plasma,[11] serum,[12] and urine[13] have been developed, among others; however, the use of MIPs in hair analysis has not been reported previously. The hair extraction method with MIPs is expected to remove hair matrix interferences, thus providing cleaner extracts than the corresponding SPE method, leading to a more sensitive and reliable analytical protocol.

Benzodiazepine detection has been reported for hair collected from living and deceased drug users by gas chromatography–mass spectrometry,[14–22] high performance liquid chromatography (HPLC)[22–24] and liquid chromatography–tandem mass spectrometry (LC–MS–MS).[25–30]

The aim of this study was to develop a molecularly imprinted solid-phase extraction (MISPE) protocol for diazepam in hair

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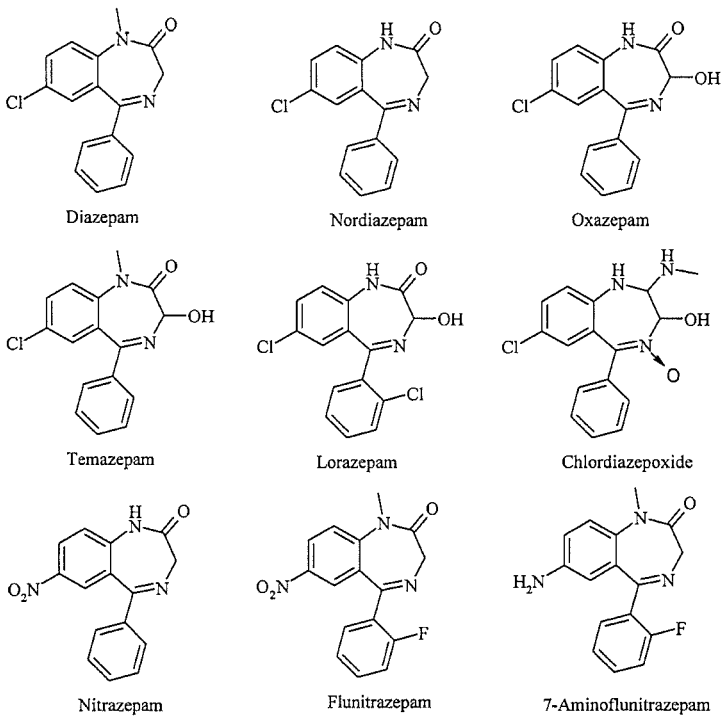

Figure 1. Chemical structures of benzodiazepine derivative drugs.

samples, based on an imprinted material reported previously by Vlatakis et al.[29] Diazepam belongs to the benzodiazepine derivative drug group and has been used as an anti-anxiety agent and muscle relaxant. Benzodiazepines are the most commonly prescribed tranquillizers in the U.K. Due to the easy availability of these drugs, drug abusers take benzodiazepines as illicit replacement drugs, sometimes together with other drugs and alcohol, either to calm themselves down or to amplify the drug-induced high. When consumed, diazepam is metabolized in the liver to produce nordiazepam via N-demethylation. Both of these compounds are converted further to oxazepam and temazepam in vivo.[31] In single-dose administration, peak plasma concentrations occur after 0.5–1.5 h. For chronic daily administration, steady-state plasma concentrations for diazepam and its metabolites are established between 5 and 14 days after the initial use. Although blood and urine samples are commonly utilized in benzodiazepine analyses, unlike hair samples, blood and urine samples only provide a short window of detection. Furthermore, collection of blood and urine samples is more invasive than the collection of hair samples, and the blood and urine samples need to be carefully collected, stored, and dispatched. Hair samples become more important when dealing with drug-facilitated crime cases, especially when the crimes are reported 24 h after consumption. In some cases, hair analysis is even accurate enough to determine drug use in intermittent users. It would be a great advantage to have a sensitive method of analysis that is able to determine not only diazepam but simultaneously also its metabolites and other benzodiazepines in hair samples.

In this study, a MISPE protocol for diazepam has been developed and tested for nine benzodiazepine drugs, including diazepam (Figure 1). The results show that the MIP is selective not only for diazepam and its metabolites but also for some other drugs from the benzodiazepine family.

EXPERIMENTAL SECTION

Materials. Diazepam for polymer synthesis was obtained from Roche (Hertfordshire, UK). Methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) were sourced from Aldrich (Steinhem, Germany), chloroform was from Rathburn (Peebleshire, UK), and 2,2′-azobisisobutyronitrile (AIBN) was purchased from Acros Organics (Geel, Belgium).

For LC–MS–MS confirmation, diazepam, nordiazepam, nitrazepam, chlordiazepoxide, temazepam, oxazepam, lorazepam, flunitrazepam, 7-aminoflunitrazepam, and the internal standards (diazepam-d_5, nordiazepam-d_5, temazepam-d_5, oxazepam-d_5, lorazepam-d_4, flunitrazepam-d_7, 7-aminoflunitrazepam-d_7) were purchased from Promochem (Teddington, UK). It was not financially viable to use the chlordiazepoxide-d_5 internal standard; therefore, oxazepam-d_5 was selected for chlordiazepoxide quantitation since its retention time (t_R) was close to the chlordiazepoxide t_R (5.1 and 6.2 min, respectively). The m/z of the nitrazepam-d_5 internal standard precursor ion is the same as the m/z of the oxazepam

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Table 1. Effect of Solvent on Diazepam Binding to the MIP and NIP

solvent	dielectric constant, ϵ	% bound MIP	% bound NIP
toluene	2.4	94.0	22.0
chloroform	4.8	72.8	29.5
dichloromethane	9.1	70.6	48.0

precursor ion ($m/z = 287$), and both have a similar t_R of 5.8 min, resulting in undesirable coelution. Therefore, oxazepam-d_5 was used as the internal standard in nitrazepam quantitation. Ammonium formate and sodium dodecyl sulfate were from Sigma-Aldrich (Dorset, UK). HPLC grade acetonitrile, formic acid, toluene, chloroform, and dichloromethane were purchased from BDH (Poole, UK).

Instrumentation. LC–MS–MS analyses of benzodiazepines were carried out using a Thermo Finnigan LCQ Deca XP Plus (Thermo Finnigan, San Jose, CA) equipped with a Surveyor HPLC system. Chromatographic separation was performed using a Gemini C18 column (150 mm × 2.0 mm i.d., 5 μm) with a guard column of the same packing as the column (4.0 mm × 2.0 mm, 5-μm) were purchased from Phenomenex (Torrance CA).

Preparation of Diazepam MIP. The MIP for diazepam was prepared using a method described by Vlatakis et al.,[30] by dissolving diazepam (0.2202 g, 0.77 mmol), inhibitor-free MAA (0.3996 g, 4.64 mmol), and inhibitor-free EGDMA (4.6004 g, 23.21 mmol) in ethanol-free chloroform (6.667 mL) in a 25 mL, thick-walled glass Kimax culture tube. AIBN (0.0838 g, 0.51 mmol) was then added and the solution sparged with oxygen-free nitrogen for 5 min while cooling in an ice bath. The sealed Kimax tube was thermostated at 4 °C, to facilitate template–monomer complex formation, and irradiated with a Blak-Ray long-wave UV lamp (model B-100A) for 24 h. Thereafter, the polymer monolith obtained was transferred to a water bath set at 60 °C for a period of 24 h, to complete the cure of the polymer. A non-imprinted polymer (NIP) was prepared in the same manner as the MIP but in the absence of template (diazepam).

The MIP and NIP monoliths were crushed, mechanically ground, and wet-sieved using acetone to deliver polymer particulates in the 25–38 μm size range. The template was extracted from the MIP by extensive washing with a mixture of methanol and acetic acid (9/1, v/v) for 24 h. The polymer particles were then dried in vacuo at 60 °C prior to use.

MISPE. The 20 μm polyethylene frits were inserted into empty 1 mL polypropylene SPE cartridges. The cartridges were connected to a vacuum manifold, and 20 mg of the MIP or NIP was slurried with acetonitrile and packed into the cartridges. The cartridges were subjected to vacuum for 30 s before insertion of a second frit on top of the sorbent bed. In order to evaluate the level of bleeding of residual template from the MIP cartridge, the MIP column was washed sequentially with 1 mL of acetonitrile containing 5, 8, 10, 15, 20, and 25% (v/v) acetic acid, starting with the eluent containing the lowest percentage of acetic acid (5%). The eluent from each washing step was collected, evaporated to dryness, and reconstituted with the mobile phase prior to analysis for diazepam using the LC–MS–MS method. It was found that the last traces of diazepam template were removed in the washing step using 10% acetic acid in acetonitrile. Therefore, 15% acetic acid in acetonitrile was used as the eluent in all subsequent elution steps.

Table 2. Recoveries of Benzodiazepines on the MIP and NIP

analyte	mean % recovery (% RSD) for $n = 5$; amount added, 50 ng MIP	NIP
7-aminoflunitrazepam	91.9 (1.5)	71.0 (7.5)
oxazepam	73.4 (8.7)	41.4 (34.3)
lorazepam	97.2 (17.0)	48.1 (22.0)
chlordiazepoxide	61.6 (13.5)	32.0 (86.8)
temazepam	89.6 (13.4)	54.3 (52.9)
flunitrazepam	39.0 (5.1)	2.3 (67.8)
nordiazepam	102.9 (9.8)	65.0 (14.3)
nitrazepam	92.3 (5.4)	60.6 (33.9)
diazepam	93.0 (1.5)	16.3 (17.1)

LC–MS–MS Analysis. LC was carried out using 3 mM ammonium formate and acetonitrile. The elution program consisted of a linear gradient (65–20%) of 3 mM ammonium formate for 13 min. The percentage of ammonium formate was decreased to 10% between 13 and 13.5 min. It was then held at 10% between 13.5 and 16.5 min before being increased back to the initial conditions (65%) between 16.5 and 20 min. The 20 μL samples were injected onto the Gemini C18 column at a flow rate of 0.3 mL min^{-1}. Ionization of analytes was performed using electrospray ionization in the positive ion mode. The nitrogen sheath and auxiliary gas flow rates and collision energy were set at the optimized conditions for each analyte.

Effect of Solvents on Diazepam Binding to Imprinted and Non-imprinted Polymers. The solvent plays an important role in the recognition step. For those cases where recognition is driven by hydrogen bonding, the interaction forces between the analyte and the polymer are diminished when increasingly polar solvents are applied. To determine the effect of solvent on the diazepam MIP, the imprinted and non-imprinted polymers were conditioned with 0.5 mL of chloroform, dichloromethane, or toluene prior to loading of 50 ng of diazepam in 1 mL of each solvent, respectively. The cartridges were then washed with 0.5 mL of the same solvent used in the loading step, before eluting with 0.5 mL of 15% (v/v) acetic acid in acetonitrile. The fractions collected after the loading (FL), washing (FW), and elution (FE) steps were blown down using a stream of nitrogen gas and reconstituted in 100 μL of the LC–MS–MS mobile phase, 20 μL of which was injected for analysis. The percentage of diazepam bound to the polymer was calculated using eq 1.

$$\% \text{ bound} = (\text{peak area for FE}/\text{peak area of } [\text{FL} + \text{FW} + \text{FE}]) \times 100 \quad (1)$$

Recoveries of Diazepam and Other Benzodiazepine Derivative Drugs. Benzodiazepine derivative drugs, namely, 7-aminoflunitrazepam, oxazepam, lorazepam, chlordiazepoxide, temazepam, flunitrazepam, nordiazepam, nitrazepam, and diazepam, were used to probe the group selectivity of the diazepam MIP (Figure 1). Samples of 20 and 50 ng of each drug were prepared in 1 mL of

Table 3. LOD and LOQ of Benzodiazepines in Hair

analyte	LOD (ng/ 30 mg)	LOQ (ng/ 30 mg)
7-aminoflunitrazepam	0.03	0.06
oxazepam	0.13	0.21
lorazepam	0.66	1.11
chlordiazepoxide	0.33	0.57
temazepam	0.39	0.63
flunitrazepam	0.78	1.32
nordiazepam	0.21	0.33
nitrazepam	0.06	0.11
diazepam	0.09	0.14

toluene. The cartridge was pre-conditioned with 0.5 mL of toluene. Each sample was passed through a cartridge, and the cartridge washed with 0.3 mL of toluene. The analytes retained on the cartridge were eluted with 0.5 mL of 15% (v/v) acetic acid in acetonitrile. The 100 ng sample of internal standard was then added after the extraction. The recovery was calculated by comparing peak area ratios obtained from extracted samples (quantitation ion/internal standard) to the peak area ratios of the same concentration of pure standards (quantitation ion/internal standard) in unextracted samples. The percent mean recovery and percent relative standard deviation (RSD) was calculated for each drug at each concentration.

Binding Capacity of Diazepam MIP. Determination of the diazepam binding capacity for the MIP cartridge was performed using the optimized procedure, with toluene as the solvent of choice. Six different concentrations of diazepam (1200, 1600, 2000, 2200, 2400, and 2600 ng) were prepared in 1 mL of toluene and extracted using six different diazepam MIP cartridges. The 100 ng internal standard was spiked after the extraction. Peak area ratios from the LC–MS–MS analyses were plotted against the diazepam concentrations to determine the maximum binding capacity value.

Cross-Reactivity of Diazepam MIP with Morphine. A 50 ng aliquot of morphine was subjected to the same extraction procedure as above, in triplicate. The eluent collected from the extraction and two non-extracted samples containing the same concentration of morphine were blown down and reconstituted in the mobile phase prior to the LC–MS–MS analyses. The percentage of morphine bound to the diazepam MIP was calculated using eq 2.

$$\% \text{ bound} = \text{peak area of extracted morphine} / \text{peak area of unextracted morphine} \times 100 \quad (2)$$

Decontamination of Hair Samples and Pre-extraction. Prior to analysis, hair samples were decontaminated and pre-extracted using a method described by Miller et al. [32] Benzodiazepines are known to be unstable in strong alkaline media;[19] therefore, a method published previously using mild alkaline incubation conditions, with good extraction efficiency for diazepam, was used.[22] The stability of the benzodiazepine drugs using these mild alkaline incubations conditions has been described previously.[32] No significant hydrolysis of the drugs was observed, and recovery values ranged from 87 to 102%, with acceptable precision. Hair samples were washed with 0.1% aqueous sodium dodecyl sulfate and sonicated for 10 min. The hair samples were then rinsed and sonicated for 10 min, twice with deionized water and twice with dichloromethane, and then left to dry in air. Washed hair samples were then weighed out and ~30 mg samples transferred into vials for the hair pre-extraction process.

In the hair pre-extraction process, 1.5 mL of methanol/25% aqueous ammonium hydroxide (20/1, v/v) was added to each vial containing a hair sample, sonicated for 1 h, and left overnight at room temperature. The extraction solvent was removed and transferred from the vial to a test tube. The hair was washed twice more with 0.75 mL of methanol/25% aqueous ammonium hydroxide (20/1, v/v), and the washings were removed and transferred to the same test tube as the first extract. The contents of the test tubes were evaporated to dryness under a stream of nitrogen gas and reconstituted with 1 mL of toluene prior to extraction with the diazepam MIP.

Limits of Detection (LOD) and Limits of Quantification (LOQ) in Hair Samples. The LOD and LOQ were determined for each drug using spiked hair. The 30 mg of blank, decontaminated hair was spiked with 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 2, and 5 ng of each drug and 100 ng of the internal standard mixture. The samples were then preextracted, extracted using the MISPE method, and analyzed by LC–MS–MS. The LOD and LOQ were determined at a signal-to-noise ratio of 3 and 5, respectively.

Application to Post-mortem Hair Samples. Three post-mortem hair samples were selected from individuals who had been shown previously to be positive for benzodiazepines in blood using a published ELISA method.[32] A 10 mg aliquot of each sample was decontaminated, preextracted, and extracted using the diazepam MIP. The samples were also screened for benzodiazepines using a validated in-house SPE method.

RESULTS AND DISCUSSION

Effect of Solvent on Diazepam Binding to Imprinted and Non-imprinted Polymers. The percentage of diazepam bound to the polymers using three different solvents in the extraction protocol is shown in Table 1. These data enable the optimal solvent for further experiments to be identified. MIPs tend to exhibit optimal molecular recognition when in the presence of the solvent that was used as the porogen during polymerization; in the present case, this was chloroform. However, the percentage of diazepam bound was slightly lower when using chloroform than toluene, suggesting that tighter binding is gained in the more apolar solvent. In other words, the solvent with the lower dielectric constant is the more effective solvent for rebinding of diazepam to the diazepam MIP.

To optimize the extraction protocol, different volumes of the washing solvent were evaluated. In addition to the assessment of pure solvents in the MISPE washing step, the washing step was also performed using 0.5 mL of 0.5, 1, and 2% acetic acid in toluene. From the results (data not shown), it was found that 0.3 mL of toluene as the washing solvent was sufficient to deliver cleaner extracts without significant loss of diazepam during the extraction process.

Recoveries of Diazepam and Other Benzodiazepine Derivative Drugs. The optimized extraction protocol was used to determine the recoveries of benzodiazepines with the MIP and NIP (Table 2). The results demonstrate that the MIP recovered

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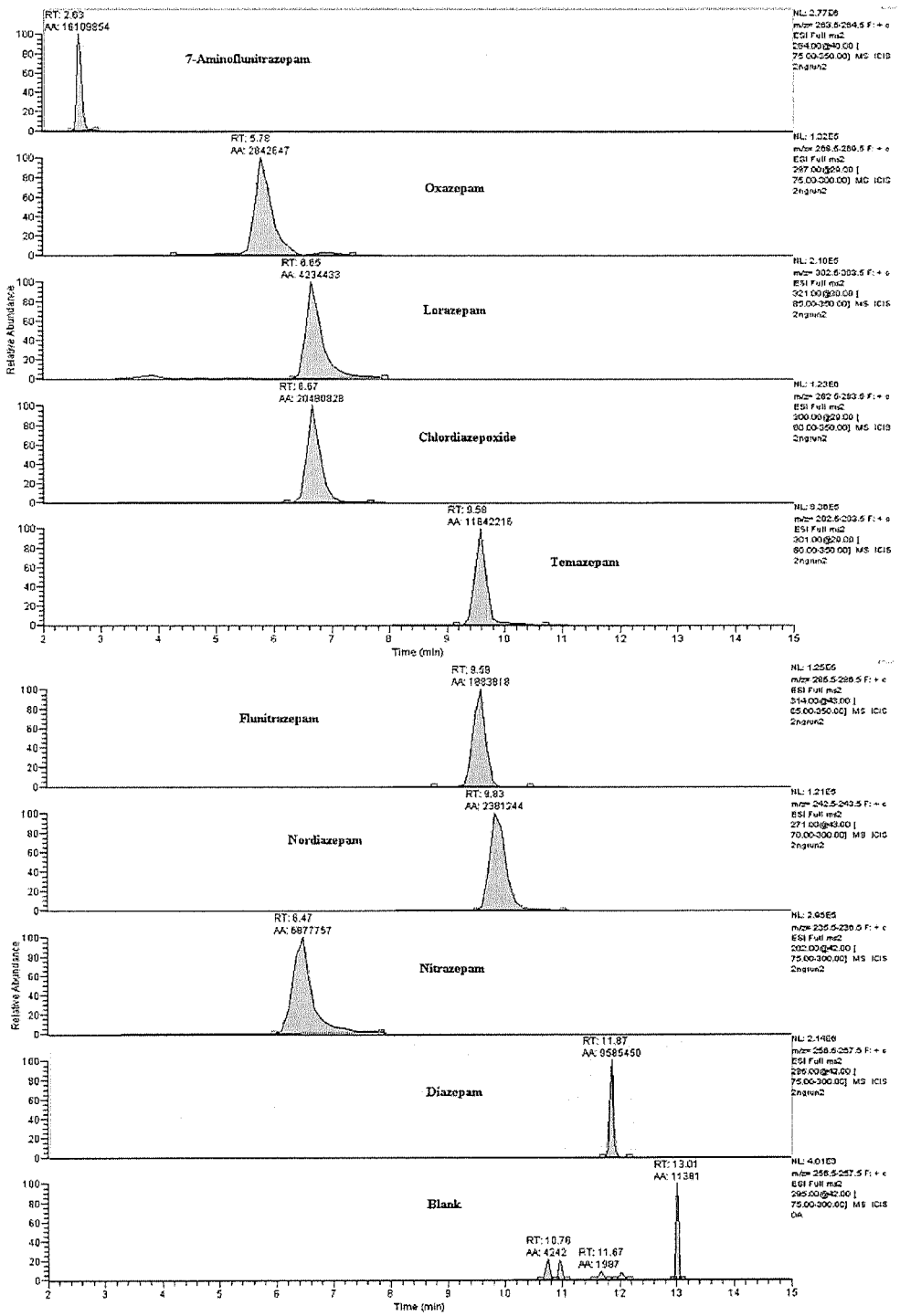

Figure 2. Chromatograms of quantitation ions of benzodiazepines in blank hair spiked at a concentration of 2 ng/30 mg, and a blank hair extract, subsequent to the MISPE step.

a high percentage of 7-aminoflunitrazepam, lorazepam, temazepam, nordiazepam, nitrazepam, and diazepam, with acceptable RSD values. Flunitrazepam and chlordiazepoxide were bound to a lesser extent, presumably due to a poorer fit in the imprinted binding

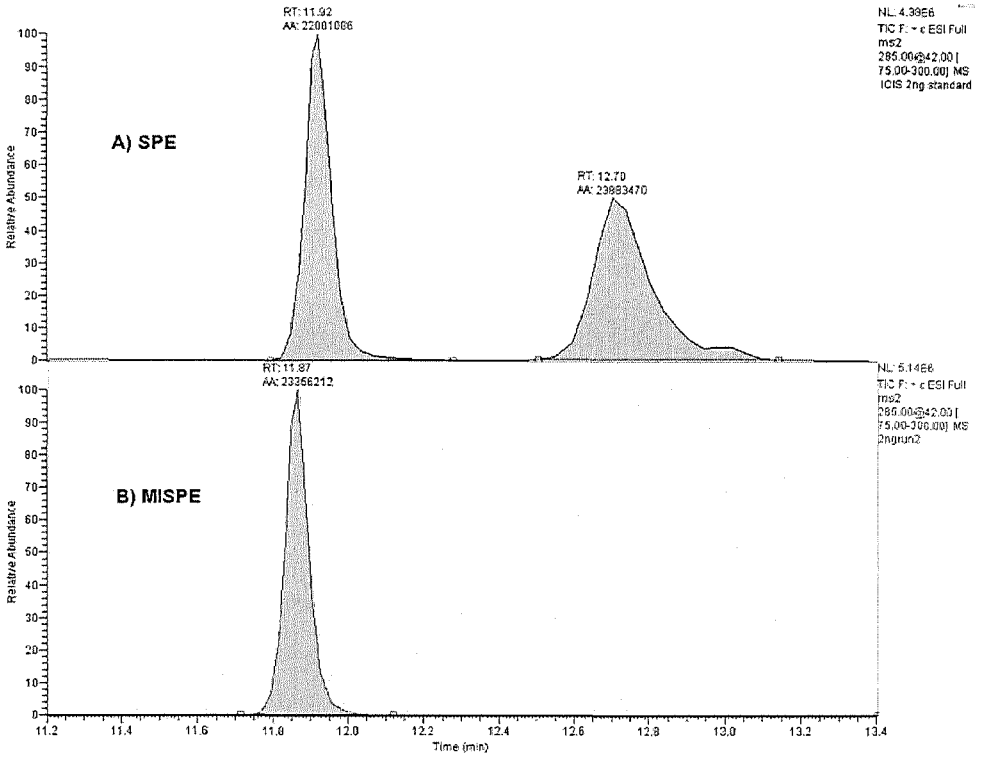

Figure 3. Chromatograms of quantitation ions of diazepam in blank hair spiked at 2 ng/30 mg of hair using (A) SPE and (B) MISPE.

Table 4. Results from the MISPE Analysis of Benzodiazepines in Hair Compared to the SPE-LC–MS–MS Analysis of the Corresponding Blood Samples

	diazepam		nordiazepam		oxazepam		temazepam	
sample	hair (ng/mg)	blood (ng/mL)	hair (ng/mg)	blood (ng/mL)	hair (ng/mg)	blood (ng/mL)	hair (ng/mg)	blood (ng/mL)
1	0.06	0.03	0.09	0.01	nd[a]	nd	nd	nd
2	0.01	0.03	0.08	0.05	nd	nd	nd	nd
3	0.67	0.31	0.31	0.53	0.18	0.12	0.15	0.05

[a] nd, not detected

pockets. The high recovery of diazepam on the MIP resulted from the excellent molecular recognition of the template molecule imparted by the imprinting process. Lower recoveries were obtained when the NIP was used as the extraction sorbent, as expected, together with high RSD values. The higher RSD values arising from use of the NIP may reflect the fact that the NIP is relatively ill-defined, in respect of its binding character, compared to the MIP.

Binding Capacity of Diazepam MIP and Cross-Selectivity with Morphine. The binding capacity of the imprinted polymer was found to be 110 ng of diazepam/mg of polymer. By using the same extraction protocol for morphine as was used for diazepam, the cross-selectivity of the MIP for morphine was found to be lower than 15%.

Limits of Detection and Limits of Quantitation in Hair Samples. The LOD and LOQ for benzodiazepines ranged from 0.03 to 0.78 ng/mg and from 0.06 to 1.32 ng/mg, respectively (Table 3). The correlation coefficients (r^2) of the calibration were >0.99 for all analytes, except for flunitrazepam, where $r^2 = 0.98$. Chromatograms of quantitation ions for all the benzodiazepines at 2 ng/30 mg, as well as the blank extract, are shown in Figure 2. From the blank extract chromatogram, it is clearly shown that no bleeding of template was observed. Furthermore, the MISPE extracts were cleaner for diazepam than for conventional SPE, showing less interference in LC–MS–MS (Figure 3).

In passing, it is worth noting that even if template bleeding were to be observed, then the group-selective binding character of the MIP would still allow for the accurate quantitation of diazepam metabolites and other members of the benzodiazepine family. Diazepam itself could be quantified by using a MISPE sorbent imprinted with one of the other members of the benzodiazepine family.

Application to Post-mortem Hair Samples. ELISA screening results showed that all three samples were positive for

benzodiazepines. Since ELISA is a semi-quantitative test, the concentrations are not quoted. It was used to identify whether the samples were positive or negative. The concentrations of benzodiazepines in the hair samples, as determined from LC–MS–MS analysis following the MISPE step, are shown in Table 4. The results were compared with the concentrations in blood, which had been determined using a validated in-house SPE and LC–MS–MS method,[32] to assess the correlation, if any, between the methods.

In Samples 1 and 2, diazepam and nordiazepam were found at low levels, while in Sample 3, diazepam and all its metabolites, namely, nordiazepam, oxazepam, and temazepam, were detected at levels typically found in chronic users.[17,19] The results show that there is good agreement with the literature methods.

CONCLUSION

In this study, a MISPE extraction method for diazepam in hair samples has been developed. Results for diazepam analysis have been found to be accurate, reproducible, and sensitive. The LOD and recovery values for diazepam by MISPE are 0.09 ng of diazepam/30 mg of hair and 93%, respectively, compared to 0.13 ng of diazepam/30 mg of hair and 69% using the SPE method described by Miller et al.[32] The method also works for other benzodiazepine drugs due to the group-selective binding nature of the MIP. The results arising from the application of this new method to post-mortem hair analysis are in excellent agreement with the ELISA method and corresponding blood sample results. The performance characteristics, in terms of sensitivity, selectivity, and cleanliness of the extracts, indicate that this method is sufficiently accurate and precise to be used for trace diazepam, diazepam metabolite, and benzodiazepine analysis in hair samples, a finding that may be of particular relevance to the investigation of drug-facilitated crime cases.

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Comparison of molecularly imprinted polymer solid-phase extraction (MISPE) with classical solid-phase extraction (SPE) for the detection of benzodiazepines in post-mortem hair samples

Abstract

This preliminary study compares the benzodiazepine hair results for ten post-mortem scalp samples using a classical solid-phase extraction (SPE) and a molecularly imprinted solid-phase extraction (MISPE) system. The hair samples selected for testing were from drug-related deaths where a positive benzodiazepine blood result was obtained. Samples were decontaminated with 0.1 % sodium dodecyl sulfate, distilled water and dichloromethane, incubated overnight in methanol/25 % aqueous ammonium hydroxide (20:1), extracted by SPE or MISPE and subsequently analysed by liquid chromatography-tandem mass spectrometry (LC-MS-MS).

Both extraction methods detected diazepam, nordiazepam, oxazepam, temazepam and nitrazepam in the samples. Diazepam was detected in a greater number of samples using MISPE due to both its lower limit of detection (LOD) and higher extraction recovery as a result of excellent molecular recognition of the template (diazepam) imparted by the imprinting process. The selective recognition of two diazepam analogues, nordiazepam and oxazepam, using MISPE was demonstrated since they were also detected in a greater number of samples. In contrast, another diazepam analogue, temazepam, was detected in a greater number of samples using SPE since the LOD using this extraction was lower than with MISPE. Overall the MISPE and SPE hair results are in good qualitative agreement.

For the samples where both extraction methods tested positive for nordiazepam, temazepam and oxazepam, the concentrations detected were always higher for SPE.

This is probably due to the MIP procedure producing extracts with fewer matrix interferences than the extracts produced using the classical SPE method.

MISPE could be used as a complementary method to classical SPE extraction for the analysis of benzodiazepine positive hair samples collected from chronic users.

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Introduction

Benzodiazepines include a large number of drugs which are prescribed as sedatives, hypnotics, muscle relaxants and anticonvulsants. The benzodiazepine drug class includes the most frequently prescribed and abused tranquillisers in the UK as they are inexpensive, effective and safe. They are, however, addictive and readily available. therefore are widely abused [1]. Drug abusers take benzodiazepines as illicit replacement drugs, sometimes simultaneously with alcohol and other drugs such as opiates, sedatives, antidepressants and neuroleptics [2] to either calm themselves down or amplify the drug-induced high. One study into drug-related deaths in the Strathclyde region of Scotland between 1995 and 1998 found that 45 % of cases tested positive for diazepam while 33 % of tested positive for temazepam [3]. An assessment of drug prevalence in impaired and fatally injured drivers in the same region during the same period found benzodiazepines to be present in 82 % of cases [4]. The Scottish Health Statistics show that diazepam is currently the most commonly prescribed benzodiazepine in Scotland [5].

Blood and urine samples are commonly analysed for benzodiazepines and, in contrast to hair samples, only provide information on drug use related to time scales of hours to a few days prior to collection. Hair is a useful source for establishing prior drug use in the weeks or even months prior to collection. In addition, collection of hair samples is easier and less invasive than the collection of blood or urine samples and hair samples can be easily stored and transported.

Benzodiazepines have been detected in hair samples from living and deceased abusers using gas chromatography-mass spectrometry (GC-MS) [6-11], high performance liquid chromatography (HPLC) [12] and liquid chromatography-tandem mass spectrometry (LC-MS-MS) [13-18]. Reported extraction methods include solid-phase extraction [2, 5, 11, 12, 17], liquid-liquid extraction [9-11, 13, 14, 16] and molecularly imprinted solid-phase extraction (MISPE) [19].

The classical solid-phase extraction method selected for this study operates by a mixed-mode cationic exchange mechanism. The sorbent is composed of C_8 chains and benzene sulfonic acid (BSA). The benzodiazepines adsorb onto the column *via* both hydrophobic and ionic attraction. In contrast, the molecularly imprinted polymer (MIP) is synthesised in the presence of a template molecule (in this study diazepam) and consists of a highly cross-linked porous polymer network. After the polymerisation process, the template is removed, leaving a polymer network with strategically positioned functional groups in binding sites that are complementary in size and shape to the template molecule. These binding sites have the potential to re-bind with the template molecule or other molecules which have a similar molecular structure to the template molecule, in a strong and selective way. The preparation of an anti-diazepam MIP has been previously described and applied in the extraction of diazepam and other benzodiazepines from hair *via* a solid-phase extraction protocol [19].

MIPs offer several advantages over natural receptors. They are cheaper, easier to prepare, more stable in extreme pH and temperature conditions and can be re-used. This has led to MIP development in various application areas such as chromatography [20, 21], catalysis [22], chemical sensors [23] and SPE [19, 24, 25]. MIPs have also been used in environmental chemistry for the extraction of river and soil pollutants [26, 27] and in toxicology for the extraction of drugs and poisons from plasma [28], serum [29] and urine [30].

The aim of this study was to compare the benzodiazepine hair results for ten post-mortem scalp samples using a classical SPE system and a MISPE system, where the imprinted sorbent shows molecular selectivity for benzodiazepines.

Materials studied, methods, techniques

Samples

The ten hair samples tested were post-mortem cases. One scalp sample was collected in each case. The samples selected for testing were cases in which the blood result was positive for benzodiazepines using a validated in-house LC-MS-MS method. Eleven negative hair samples were obtained from volunteers and post-mortem case samples. All samples were wrapped in aluminium foil and stored at room temperature prior to analysis.

Apparatus

LC-MS-MS

The set-up consisted of a Surveyor high performance liquid chromatography (HPLC) system with a LCQ™ Deca XP Plus (Thermo Finnigan, San Jose CA, USA) ion trap mass spectrometer. HPLC was performed on a Gemini C18 column (150 mm L x 2.0

mm ID, 5 μm particle size), fitted with a guard column with identical packing material (4 mm L x 2.0 mm ID) (Phenomenex, Torrance, CA).

Chemicals and Reagents

Methanol, acetonitrile, ammonium hydroxide, cyclohexane, ethyl acetate, formic acid, dichloromethane, toluene and propan-2-ol were purchased from BDH (Poole, UK) and were of analytical grade. Ammonium formate and sodium dodecyl sulfate were purchased from Sigma-Aldrich (Dorset, UK). 7-Aminoflunitrazepam, flunitrazepam, oxazepam, lorazepam, chlordiazepoxide, temazepam, diazepam, nordiazepam, nitrazepam, 7-aminoflunitrazepam-d7, flunitrazepam-d7, oxazepam-d5, lorazepam-d4, temazepam-d5, diazepam-d5 and nordiazepam-d5 were obtained from Promochem (Teddington, England).

An anti-diazepam imprinted polymer was synthesised according to a previously published study [31]. For MIP synthesis, diazepam was obtained from Roche (Hertfordshire, UK). Methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) were from Aldrich (Steinheim, Germany), chloroform from Rathburn (Pebbleshire, UK) and 2, 2'-azobisisobutyronitrile (AIBN) from Acros Organics (Geel, Belgium).

Methods

Washing of Hair Samples

The root-0.5 cm section was removed from each hair sample where roots were present. The remaining hair was cut into 2-3 mm segments using a pair of clean scissors. The samples were washed with 1 mL of 0.1 % aqueous sodium dodecyl sulfate with a 10 minute ultrasonication, 2 x 1 mL deionised water with a 10 minute ultrasonication and 2 x 1 mL dichloromethane with a 10 minute ultrasonication. The washings were not

analysed since the likelihood of benzodiazepine external contamination is very low. Each sample was then split into two separate vials and weighed for extraction by both SPE and MISPE procedures. Blank hair was also washed using this procedure prior to spiking to produce the calibrators for the two procedures.

LC-MS-MS

Pre-extraction

An alkaline pre-extraction, with good diazepam extraction efficiency from hair, was used [32]. The benzodiazepines were found to be stable using these incubation conditions with recoveries ranging from 87-102 % [18]. Furthermore, ammoniated methanol was found to produce slightly higher recoveries than methanol alone for diazepam, oxazepam and flunitrazepam in another study [12].

1.5 mL of methanol/ 25 % aqueous ammonium hydroxide solution (20:1) and 100 ng internal standard was added to each calibrator and sample, left to ultrasonicate for one hour and then stored at room temperature overnight. The solvent was removed from the vials and transferred to a test tube. The hair was washed twice more with 0.75 mL methanol/25 % aqueous ammonium hydroxide (20:1) and the washings removed and transferred to the corresponding test tubes. The contents of the test tubes were evaporated to dryness under nitrogen and reconstituted in either 1 mL of phosphate buffer for SPE or 1 mL toluene for MISPE [18, 19].

Conventional SPE Method

World Wide Monitoring Clean Screen® columns (ZSDAU 020) were conditioned sequentially with 3 mL methanol, 3 mL distilled water and 1 mL of phosphate buffer (0.1M, pH 6.0). The vortexed samples were loaded onto the columns and allowed to drip through without the application of a vacuum. The columns were washed

sequentially with 2 mL distilled water, 2 mL 20% acetonitrile in phosphate buffer (0.1 M, pH 6.0), 2 mL cyclohexane and 2 mL distilled water. The columns were dried for five minutes under full vacuum after the second stage of the wash step, for one minute after the third stage of the wash step and for five minutes after the final stage of the wash step. The analytes were eluted using 1.5 mL 2 % ammoniated ethyl acetate (followed by a two minute drying step on full vacuum) and 1.5 mL dichloromethane/isopropanol/ammonium hydroxide (78:20:2). The eluted samples were blown down under nitrogen and reconstituted with 100 µL of the mobile phase initial conditions. 20 µL was injected for analysis.

MISPE Method

Diazepam MIP cartridges were prepared as detailed in another study [19]. The cartridges were conditioned using 0.5 mL of toluene. The vortexed samples were added to the columns and allowed to drip through without the application of a vacuum. The columns were washed with 0.3 mL of toluene and eluted using 0.5 mL 15 % (v/v) acetic acid in acetonitrile. The eluted samples were blown down under nitrogen and reconstituted with 100 µL of the mobile phase initial conditions. 20 µL was injected for analysis.

LC-MS-MS Conditions

The column was maintained at 25 °C. The mobile phase consisted of 3 mM ammonium formate + 0.001% formic acid in water (A) and acetonitrile (B) at a flow rate of 0.3 mL/min. The initial gradient conditions were 65 % A, decreasing to 20 % A after 13 minutes. The mobile phase composition was 10 % A from 13.5-16.5 minutes. Initial conditions were restored from 16.5-20 minutes and the system allowed sufficient time to equilibrate. The total run time was 20 minutes. A divert valve taking LC flow to waste was used for the last 7 minutes of the run to preserve the MS source.

All mass spectral data was collected in electrospray positive ion mode. The capillary temperature, sheath and auxiliary gas flow rates and collision energies were optimised for each analyte. The probe voltage used was 4.5 kV. Internal standard data was collected in selected ion monitoring (SIM) mode and analytes were identified on the basis of their retention time and full MS-MS spectra. The product ion ratios were monitored to gain further qualitative identification data. The optimum tuning parameters, precursor and product quantitation ions are shown in Table 1.

Validation methods

The percentage extraction recovery was determined by comparing the analysis of extracted (n=5) and non-extracted spiked samples (n=2) at 50 ng.

The LOD and LLOQ values were calculated statistically as the intercept of the calibration graph plus three times the standard error of the line and the intercept of the calibration graph plus five times the standard error of the line respectively [18, 19]. The percentage extraction recovery, LOD and LLOQ values for the benzodiazepines are reported in Table 2.

Results

The chemical structures of the five benzodiazepines detected in the hair samples are shown in Figure 1. The MIP produced was selective for not only diazepam (the template) but also for nordiazepam, oxazepam, temazepam and nitrazepam because they are close structural analogues of diazepam.

Benzodiazepines were detected in nine samples using the MISPE method and in seven samples using the classical SPE method. No benzodiazepines were detected by either extraction method in one sample.

Diazepam was detected in four samples at low levels using MISPE where there was none detected using SPE. Nordiazepam and oxazepam were also detected at low levels using MISPE where none was detected using SPE (samples 4 and 2 respectively). The results for one sample (sample 10) did however show a significantly lower oxazepam concentration detected using MISPE compared to SPE. Temazepam was detected in two samples by SPE where none was detected by MISPE. The nitrazepam results for the MISPE and SPE methods were very similar.

The SPE and MISPE hair testing results are provided in Table 3, along with the corresponding blood concentrations. In general, the hair results are in good qualitative agreement using both extraction methods.

Discussion

The MISPE demonstrated higher percentage extraction recoveries for six out of nine analytes whereas the SPE achieved lower LOD values for six out of nine analytes.

In this study, benzodiazepine concentrations have been measured in ten post-mortem scalp hair samples using SPE and MISPE sample preparation methods. The results show that diazepam was the most frequently detected, followed by its metabolite nordiazepam. In general, benzodiazepine levels were in the ranges reported in the literature [2, 6]. Nordiazepam was always detected at a higher concentration than diazepam, a finding that is consistent with other studies [6, 10]. Oxazepam was detected only in two samples. A possible reason for this low detection rate may be that

oxazepam has a relatively short half-life compared to nordiazepam, for example, and is a polar compound which may not incorporate readily into hair [12] and could be present at low levels as a diazepam metabolite, rather than as the parent drug. Temazepam levels have not been reported widely in hair. In this study, the levels detected were within a relatively narrow concentration range of 0.16-0.24 ng/mg. Nitrazepam levels detected in one sample by MISPE and SPE were higher than reported in another study [2].

Overall, it is difficult to compare directly the benzodiazepine levels detected in our study with those reported by other authors because of the broad range of extraction and analytical methods used. It is also difficult to interpret the benzodiazepine levels detected in the hair since the amount and type of drug taken over the months prior to death is not known in the majority of cases. As well as potentially being present in hair samples due to diazepam metabolism, nordiazepam, temazepam and oxazepam can also be ingested as parent drugs or they could be present as an association with both metabolism and parent drug use.

Information contained in the police reports stated that sample 6 was from an individual who had been prescribed Valium® in the past; sample 8 was from an individual who had overdosed previously on Valium® and ecstasy; and sample 10 was from an individual who had been prescribed diazepam. The hair results for these samples supports diazepam use in the months prior to death.

Both the MISPE and SPE methods were validated [18, 19] for the nine benzodiazepines shown in Table 1, however only diazepam, its metabolites and nitrazepam were detected in the samples. The greater number of diazepam and nordiazepam positive samples detected using MISPE could be attributed to the higher extraction recovery and lower

LOD values obtained using this protocol compared to SPE. Conversely, temazepam was detected in less samples using MISPE compared to SPE due to the lower extraction recovery and LOD value.

In the samples which tested positive for diazepam using both extraction methods, the concentration detected using MISPE was generally similar to those detected using SPE. The excellent molecular recognition of the template molecule by the MIP resulting in a higher extraction recovery and lower LOD meant that diazepam was detected in four samples at low levels where SPE detected none. For the samples where both extraction methods tested positive for nordiazepam, temazepam and oxazepam, the concentrations detected were always higher for SPE. Interestingly, oxazepam was detected in one sample using MISPE where none was detected using SPE despite its slightly lower LOD. Nitrazepam levels detected by the MISPE and SPE methods were comparable.

Although the MISPE and SPE results are in good qualitative agreement, there are some quantitative differences between the two extraction methods, particularly for diazepam metabolites. Concentration differences of this type can be due to systematic bias or else they may reflect some degree of selectivity by the MIP between the analyte and internal standard during the extraction process. Taking the classical SPE method as the Gold Standard procedure, the new MISPE method shows in most cases a negative bias which, in percentage terms, is high in some cases. The bias is not completely systematic as the bias % varies with concentration. Most of the large differences relate to cases in which the concentrations measured are low and close to the LLOQ.

The most likely explanation is that the MIP procedure results in extracts with fewer matrix interferences than the SPE procedure (Figure 2), and that these interferences augment the peak areas in the product ion chromatograms obtained for classical SPE

extracts. This implies that the classical procedure may show lower selectivity than the MIP method.

The results show that the MISPE method is selective for diazepam metabolites and other benzodiazepine analogues. It detected diazepam and oxazepam in samples which tested negative using the classical SPE approach. Overall, the MISPE method appears to be significantly more streamlined than the SPE method, providing a simpler and more time efficient procedure.

Conclusion

This preliminary study into the use of a diazepam MIP for the detection of benzodiazepines in post-mortem hair case samples has shown that it can be applied successfully for this purpose. MISPE detected a higher number of diazepam positive case samples than the classical SPE because of the higher extraction recovery and lower LOD using the MISPE method as a result of the excellent molecular recognition of the template molecule (diazepam) imparted by the imprinting process. The MISPE has demonstrated selectivity for other benzodiazepine analogues and detected nordiazepam and oxazepam in two samples where SPE detected none. The MISPE was, however, not as selective for temazepam as the SPE. Nordiazepam, oxazepam and temazepam concentrations, when detected using both extraction methods, were higher using the classical SPE method. The most likely explanation for this is that the MISPE method has a higher selectivity than the SPE method, with fewer matrix interferences.

Further work is required to investigate the possibility of mixing two or more MISPE sorbents in one cartridge to detect a particular drug class and also the ability of the MISPE to detect benzodiazepines in single dose hair samples.

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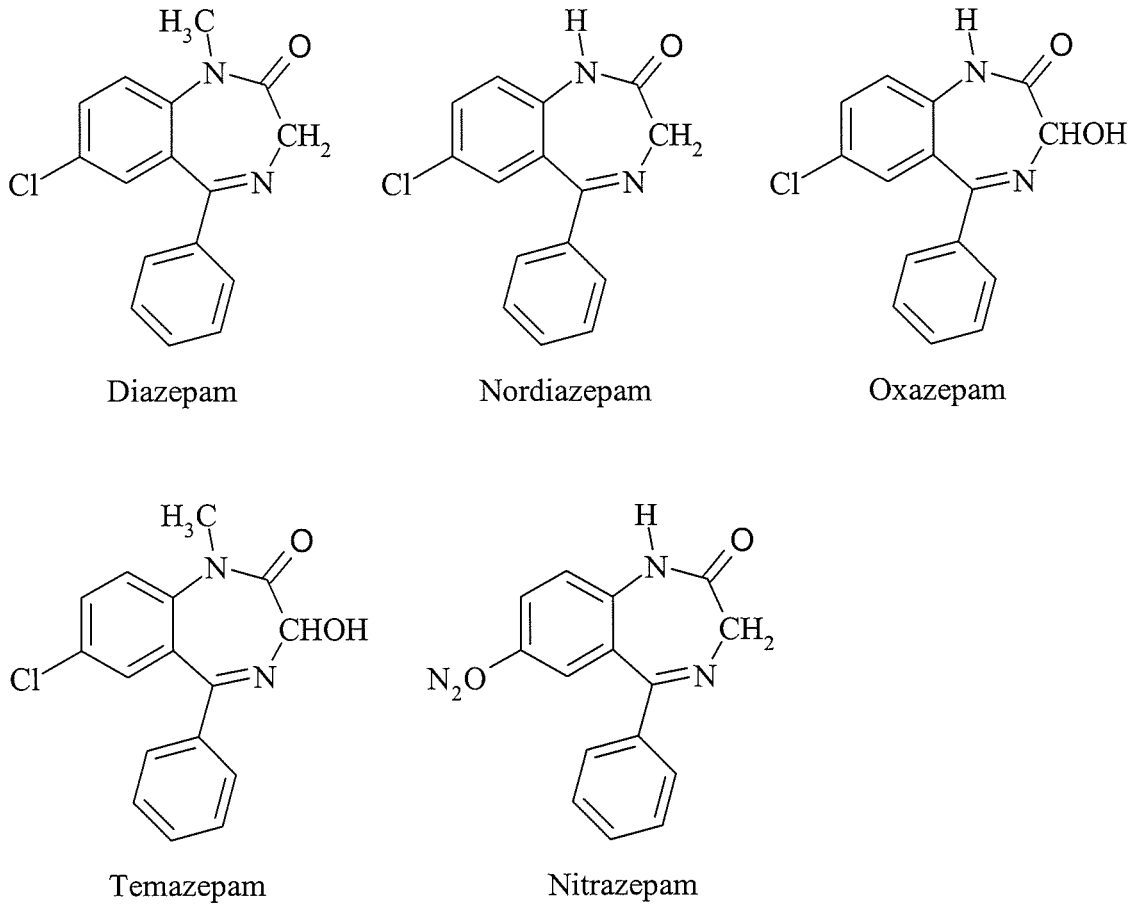

Figure 1: Chemical structures of the five benzodiazepines detected in hair samples after extraction by MISPE and SPE

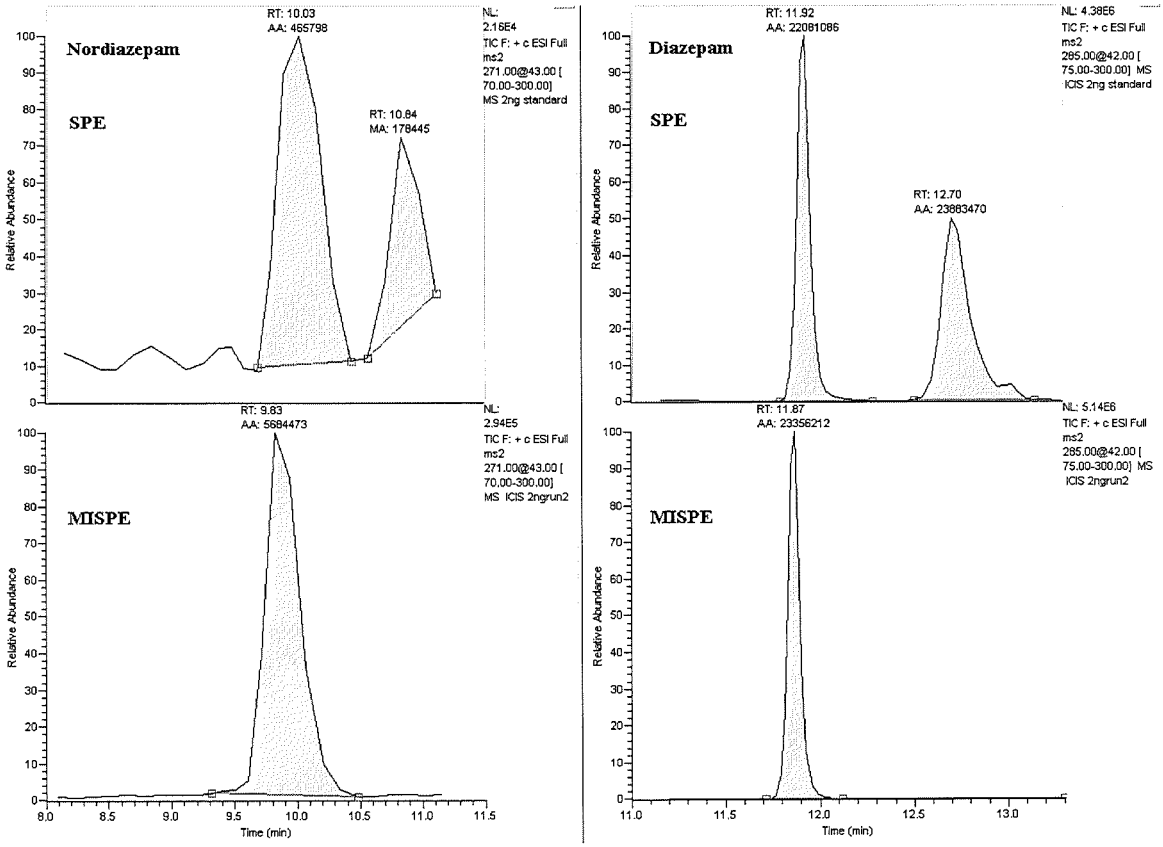

Figure 2: Chromatograms of the SPE and MISPE extracts showing LC-MS-MS interference in the nordiazepam and diazepam scan segment for the SPE extract only

Table 1 – Optimum tuning parameters, precursor and product ions for each analyte

Analyte	Sheath Gas (AU)	Auxiliary Gas (AU)	Capillary Temperature (°C)	Collision Energy (%)	Precursor ion (MH+) m/z	Product ions m/z
7-aminoflunitrazepam	20	20	280	40	284	264*, 256
Chlordiazepoxide	20	20	300	29	300	283*, 241
Diazepam	20	20	300	42	285	257*, 222
Flunitrazepam	20	20	280	43	314	286*, 268
Lorazepam	30	20	290	30	321	303*, 275
Nitrazepam	30	20	300	42	282	254, 236*
Nordiazepam	20	15	300	41	271	243*, 140
Oxazepam	20	20	300	29	287	269*, 241
Temazepam	20	20	300	29	301	283*, 255

*Quantitation ion

Table 2: MISPE and SPE hair results *vs* blood analysis results

Case Sample Number	Diazepam			Nordiazepam			Oxazepam			Temazepam			Nitrazepam		
	Hair (ng/mg)		Blood (mg/L)	Hair (ng/mg)		Blood (mg/L)	Hair (ng/mg)		Blood (mg/L)	Hair (ng/mg)		Blood (mg/L)	Hair (ng/mg)		Blood (mg/L)
	MISPE	SPE		MISPE	SPE		MISPE	SPE		MISPE	SPE		MISPE	SPE	
1	0.06	-	0.03	0.09	0.28	0.01	-	-	-	-	0.22	-	-	-	-
2	0.68	0.65	0.72	0.97	1.20	1.42	0.06	-	0.08	0.17	0.24	0.13	0.23	0.24	-
3	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-
4	-	-	0.03	0.08	-	0.05	-	-	-	-	-	-	-	-	-
5	0.03	-	0.15	0.08	0.27	0.2	-	-	0.02	-	-	0.02	-	-	-
6	0.46	0.46	0.11	-	-	-	-	-	-	-	-	-	-	-	-
7	0.06	-	0.04	0.19	0.31	0.08	-	-	-	0.16	0.22	-	-	-	-
8	0.08	0.03	0.02	0.12	0.43	0.04	-	-	-	-	-	-	-	-	-
9	0.02	-	0.15	-	-	0.07	-	-	-	-	-	0.01	-	-	-
10	0.50	0.06	0.06	0.12	0.28	0.16	0.09	0.59	-	-	0.23	-	-	-	-

'-' = not detected

Table 3: Recovery, LOD and LLOQ for MISPE and SPE

Analyte	Mean % Recovery (% RSD) for n=5 at 50 ng		LOD (ng/mg hair)		LOQ (ng/mg hair)	
	MISPE	SPE	MISPE	SPE	MISPE	SPE
7-Aminoflunitrazepam	92 (2)	55 (10)	0.03	0.14	0.06	0.23
Chlordiazepoxide	62 (14)	63 (5)	0.33	0.07	0.57	0.13
Diazepam	93 (2)	69 (2)	0.09	0.13	0.14	0.22
Flunitrazepam	39 (5)	89 (6)	0.78	0.30	1.32	0.50
Lorazepam	97 (17)	94 (10)	0.66	0.62	1.11	1.02
Nitrazepam	92 (5)	91 (9)	0.06	0.03	0.11	0.05
Nordiazepam	103 (10)	82 (5)	0.21	0.24	0.33	0.41
Oxazepam	73 (9)	83 (8)	0.13	0.11	0.21	0.19
Temazepam	90 (13)	88 (8)	0.39	0.09	0.63	0.16